PLANT PROTEINACEOUS INHIBITORS OF PROTEINASES AND α-AMYLASES

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INTRODUCTION

Plant proteins which are inhibitory towards various types of enzymes from a wide range of organisms have been extensively studied for many years. Proteinase inhibitors have received particular attention and accordingly a number of reviews concerning their structure, activity, evolution, possible physiological roles and nutritional properties have appeared regularly in the literature (Ryan, 1973, 1981, 1984; Laskowski and Kato, 1980; Richardson, 1981; Boisen, 1983). Recent technical advances in molecular biology have accelerated the output of information about these inhibitors to the extent that entirely new types have been uncovered and previously unsuspected relationships have been established. These developments justify the present review that will emphasize the novel aspects, glossing over many important topics that have been adequately covered before. Among the most striking recent findings is the structural and evolutionary relationships of different α-amylase inhibitors with different types of proteinase inhibitors, which is the reason for their joint consideration in this survey.

The inhibitors have been grouped for our present purposes as listed in Table 1, following an eclectic criterion: the first nine groups are true protein families, based on sequence homology, and the last two are based on the mechanistic classes of the enzymes inhibited because not enough sequence information is available, and therefore may represent more than one protein family. A considerable number of reports deal with inhibitors that have not been sufficiently characterized to discern whether they belong to any of the listed groups or represent new types. These cases have not been comprehensibly included in this review.

Table 1. Groups of protein inhibitors of proteinases and α-amylases

Inhibitor group		Enzymes inhibited
1.	Soybean trypsin inhibitor (Kunitz) family	Serine proteinases and endogenous α-amylases
2.	Bowman-Birk inhibitor family	Serine proteinases
3.	Cereal trypsin/α-amylase inhibitor family	Serine proteinases and heterologous α-amylases
4.	Potato inhibitor I family	Serine proteinases
5.	Potato inhibitor II family	Serine proteinases
6.	Squash inhibitor family	Serine proteinases
7.	Barley protein Z/α_1-antitrypsin family	Serine proteinases
8.	Ragi I-2/maize bifunctional inhibitors family	Serine proteinases and heterologous α-amylases
9.	Carboxypeptidase A, B inhibitor family	Metallo-carboxypeptidases
10.	Thiol-proteinase inhibitors	Endogenous and heterologous thiol-proteinases
11.	Cathepsin D and Pepsin inhibitors	Carboxyl-proteinases

All the serine proteinase inhibitors treated here seem to obey a standard mechanism, which has been thoroughly studied from different points of view (see Laskowski and Kato, 1980), including the detailed elucidation by X-ray crystallography of the three-dimensional structure of several enzyme-inhibitor complexes, as reviewed recently by Read and James (1986). In summary, the inhibitors are highly specific substrates for their target enzymes, which undergo a limited and extremely slow proteolysis, so that the system behaves as if the free enzyme and the inhibitor were in simple equilibrium with the enzyme/inhibitor complex. On the surface of each inhibitor there is at least one reactive bond (P1–P'1) which interacts with the active site of the enzyme. An inhibitor molecule which has undergone hydrolysis of its reactive bond is as active as the unhydrolised inhibitor and is able to form a stable complex with the enzyme. In most, but not all, of these inhibitors, the reactive site peptide bond is encompassed in at least one disulphide loop, which ensures that during conversion of the original to the modified inhibitor the two peptide chains cannot dissociate. The nature of the amino acid residue at the carboxyl side, P1 position, generally determines the proteinase inhibited: Lys or Arg for trypsin-like enzymes; Phe, Tyr, or Leu for chymotrypsin; and Ala for elastase. The amino side of the peptide bond, the P'1 position, does not seem to be involved in determining specificity (Laskowski and Kato, 1980).

More than one reactive site is sometimes present in a single polypeptide chain, in which case more than one enzyme molecule of the same or different specificity can be simultaneously inhibited by a single inhibitor molecule. In some of these cases, it is fairly obvious that the multiple reactive sites are associated with multiple protein domains that must have been generated from an ancestral domain by internal gene duplications, while in other cases a convergent evolutionary process cannot be excluded.

In subsequent pages, we will first deal with the different inhibitor groups, and then, their possible implication in plant metabolism, plant protection, and human and animal nutrition will be addressed.

SOYBEAN TRYPSIN INHIBITOR (KUNITZ) FAMILY

The crystallization of a trypsin inhibitor from soybean (STI) and of its complex with trypsin, which was carried out over forty years ago by M. Kunitz (1945, 1946, 1947a,b, 1949), was one of the major achievements in the early stages of research on protein inhibitors from plants. Further studies concerning its amino acid sequence (Koide and Ikenaka, 1973a,b; Koide et al., 1973), its three-dimensional structure (Sweet et al., 1974), and its mechanism of interaction with the enzyme (Sealock and Laskowski, 1969; Kowalski et al., 1974; Kowalski and Laskowski, 1976a,b; Hunkapiller et al., 1979; Baillargeon et al., 1980) made STI the first plant inhibitor to be well characterized. In spite of this early start, the identification of further members of the STI family in other species has been a rather slow process that has recently acquired momentum with the identification of a number of new inhibitors and, especially, with the realization that the inhibitors of subtilisin/endogenous α-amylase from cereals are indeed homologues of STI (Hejgaard et al., 1983; Svendsen et al., 1986; Maeda, 1986).

Distribution and Inhibitory Properties

Inhibitory proteins with M_r of about 20,000, two disulphide bridges and similar amino acid composition to that of STI have been identified in a wide range of species. Although STI appears as a single form for which two additional alleles have been found, mixtures of numerous isoforms with different inhibitory properties are present in many species (Table 2). A majority of the identified inhibitors are specific for either trypsin or chymotrypsin, with either weak or null activity for the second enzyme, while a few are about equally effective against both enzymes. Some members of this family are only weak inhibitors and it can be speculated that they might be active against other, unidentified enzymes. Only one of

Table 2. Distribution and inhibitory properties of members of the STI (Kunitz) family

Species	Inhibitor	Specificity*	Inh/Enz	Selected references
Papilionideae				
Psophocarpus tetragonolobus (winged bean)	WTI1A,1B,2,3	T-s	1:1	Yamamoto et al.1983
	WTCI1	T-s, Ch-s	1:1	Shibata et al. 1986
	WCI1	Ch-s	1:1	
	WCI2,3	Ch-s	1:2	
	WCI4	Ch-w		
Erythrina latissima	DE1	Ch-s		Joubert et al. 1985
	DE3	T-s	1:1	
E. cristagalli	DE1,8	T-s, Ch-w	1:1	Joubert and Sharon, 1985
	DE2,4	Ch-s	1:1	
	DE3	Ch-s	1:2	
E. corolladendron	DE1,5	Ch-s	1:1	
	DE6,7	T-s, Ch-w	1:1	
	DE8	T-s	1:1	
E. acanthocarpa	DE1	T-s, Ch-w		
	DE2	Ch-s		
E. caffra	DE1	T-s, Ch-s		
	DE2	Ch-s		
	DE3	T-s, Ch-w		
	DE4	T-s		
E. humeana	DE3	T-s, Ch-w		
E. lysistemon	DE1	Ch-s		
	DE2-4	T-s, Ch-w		
E. seyheri	DE1,3,5	T-s, Ch-w		
	DE2,4	Ch-s		
Glycine maxima	STIa,b,c	T-s	1:1	Kim et al. 1985

these inhibitors, DE3 from *Erythrina latissima*, has also been shown to inhibit tissue plasminogen activator (Joubert et al., 1985). The cereal inhibitors are isoform mixtures active against subtilisin and endogenous α-amylases (Warchalewski, 1977a,b; Yoshikawa et al., 1976;

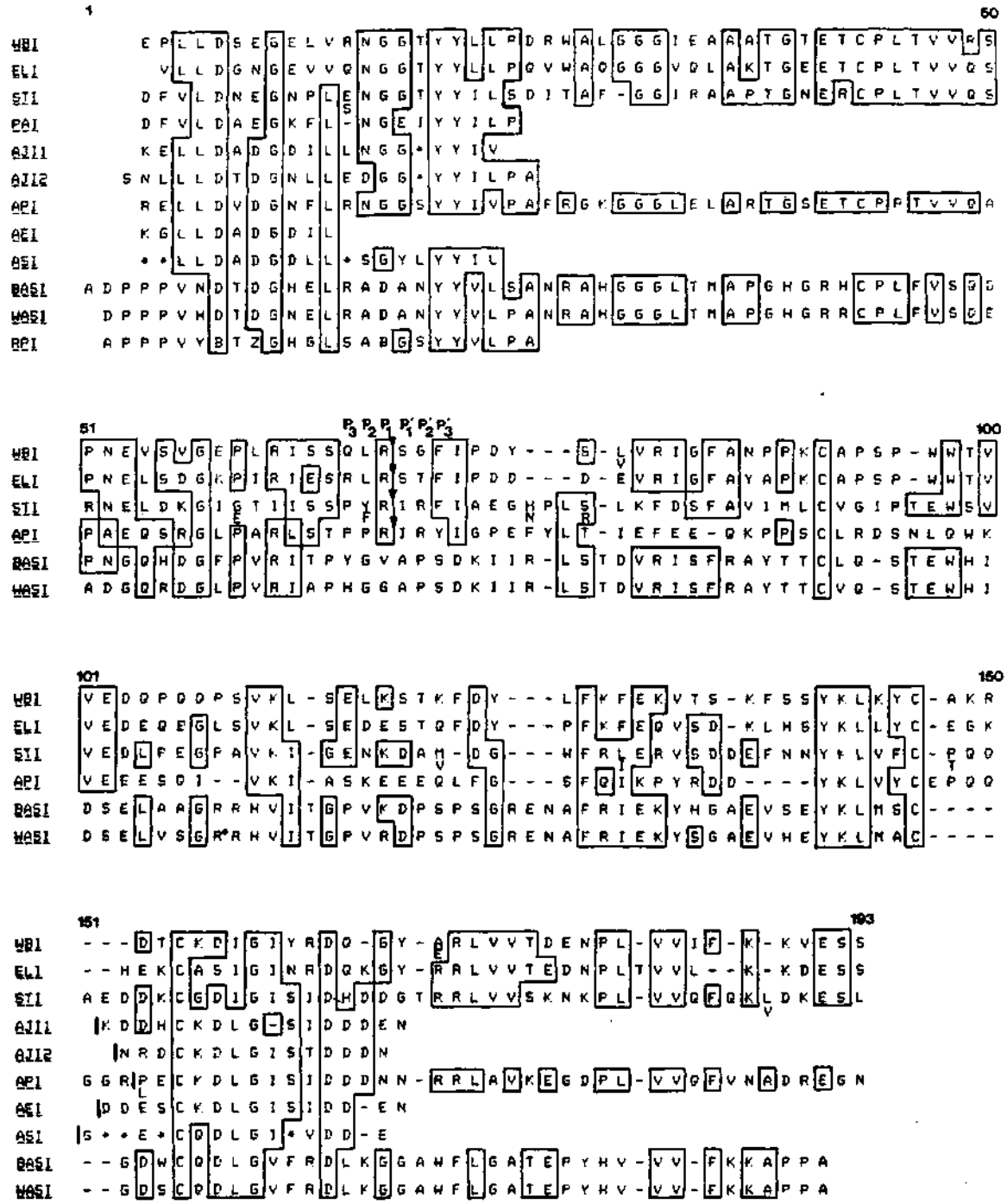
Table 2. Continued

Species	Inhibitor	Specificity*	Inh/Enz	Selected references
Cesalpinoideae				
Peltophorum africanum	DE1	T-s		Joubert 1981
Mimosideae				
Adenanthera pavonina (carolina tree)	DE1-8	T-s, Ch-s	(DE5)1:1	Richardson et al. 1986 Sudhakar Prabhu and Pattabiranam 1980
Albizzia julibrissin (silk tree)	AII,III BI,II	T-s, Ch-s Ch-s	1:1	Odani et al. 1979
Acacia elata		T-s		Kortt and Jermyn 1981
A. sieberana		T-s, Ch-s	1:1	Joubert 1983
Gramineae				
Hordeum vulgare (barley)	BASI	S-s, αA-s	1:1+1	Svendsen et al.1986
Triticum aestivum (wheat)	WASI	S-s, αA-s	1:1+1	Mundy et al. 1984 Maeda 1986
Secale cereale (rye)	RASI	S-s, αA-s		Weselake et al. 1985 Mosolov and Shulgin 1986

*T, trypsin; Ch, chymotrypsin; S, subtilisin; αA, α-amylase; s, strong; w, weak

Weselake et al., 1983a,b; Mundy et al., 1983; Hejgaard et al., 1983). More specifically, the α-amylase 2 isozymes from barley, wheat, rye and oats are inhibited, while the α-amylase 1 isozymes are not (Mundy et al., 1984). Amylases from sorghum, rice, saliva, pancreas, *Aspergillus oryzae* or *Bacillus subtilis* were found to be insensitive (Mundy et al., 1984). Not included in Table 2 are certain inhibitors whose classification as Kunitz-type is still uncertain, although their M_rs and amino acid compositions are compatible with such a classification. This is the case for the trypsin inhibitor CPPTI-fm

from *Cucurbita pepo* (Pham et al., 1985), the kallikrein inhibitors from potatoes (Hojima et al., 1973), a protease inhibitor from spinach leaves (Satoh et al., 1985), a trypsin inhibitor from white mustard (Menegatti et al., 1985), and the trypsin inhibitors from the tubers of taro (Ogata and Makisumi, 1984). The latter are of particular interest because their dimeric nature has been well established (Ogata and Makisumi, 1985).



The interaction between the Kunitz-type inhibitors and those enzymes against which they show strong inhibition is generally stoichiometric, as summarized in Table 2. All reported trypsin inhibitors form a 1:1 complex with the enzyme and those few that are also effective against chymotrypsin form a 1:1 complex with this enzyme, which can be displaced from the complex by trypsin. Most of the inhibitors that only inhibit chymotrypsin form a 1:1 complex, but certain inhibitors from *Psophocarpus* and *Erythrina* are able to form 1:2 complexes. In this context, it is of interest to note that Bosterling and Quast (1981) demonstrated the ability of STI to bind two molecules of chymotrypsin, an enzyme that is not inhibited by it, while only one molecule of chymotrypsin was bound if the inhibitor had been previously incubated with trypsin. The cereal inhibitors are able to simultaneously bind one molecule of subtilisin and one molecule of α-amylase (Mundy et al., 1983, 1984; Weselake et al., 1983a,b; Halayko et al., 1986).

The inhibitors from species of the Mimosidae subfamily, such as *Acacia*, *Albizzia*, and *Adenanthera* spp., are composed of two disulphide- linked chains, whereas the remaining inhibitors from Leguminoseae and those from Triticeae are single-chained. It seems likely, from their alignment with the single-chain inhibitors, that the two chains are originated from a single chain precursor (see Fig. 1).

Figure.1. (opposite) Alignment of amino acid sequences of members of the soybean trypsin inhibitor (Kunitz) family. WBI is a trypsin inhibitor from winged bean, Psophocarpus tetragonolobus *(Yamamoto et al., 1983); ELI is trypsin inhibitor DE3 from* Erythrina latissima *(Joubert et al., 1985); STI is the Kunitz trypsin inhibitor from soybean (Kim et al., 1985); PAI is a trypsin inhibitor from* Peltophorum africanum *(Joubert, 1981); AJI1 and AJI2 are partial sequences of the two-chained inhibitors AII and BII from* Albizzia julibrissin *(Odani et al., 1979); API is the two-chained trypsin inhibitor DE5 from* Adenanthera pavonina *(Richardson et al., 1986); AEI and ASI are partial sequences of the two-chained trypsin inhibitors from* Acacia elata *(Kortt and Jermyn, 1981) and* Acacia sieberana *(Joubert, 1983), respectively; BASI and WASI are inhibitors of subtilisin and of endogenous α-amylases from barley (Svendsen et al., 1986) and wheat (Maeda, 1986), respectively; RPI is a rice inhibitor (Kato et al., 1972; cited by Mundy et al., 1984). Vertical arrows (▼) indicate the reactive bonds of the trypsin inhibitors. Gaps introduced for the alignment are indicated (-). The N-terminal positions of the second chain of the two-chained inhibitors are indicated by a vertical line (▌). Conserved positions are boxed. Unidentified residues are indicated by an asterisk (*).*

Structure and Evolution

Available sequence data for homologues of STI (Kunitz) in different taxa are summarized in Fig. 1, and the homology matrix derived from it is presented in Table 3, where only common segments of the sequences were used to calculate percent homology in the cases in which the sequences were incomplete. As expected, higher homologies are observed within each of the Leguminosae sub-families and among the cereal inhibitors, than between these taxonomic groups, with the notable exception of STI itself, which seems to be significantly closer to the inhibitor from *Peltophorum* (sub-family Caesalpinoideae) than to those of *Erythrina* and *Psophocarpus*, which belong to the Papilionaceae sub-family, together with *Glycine* (soybean). Inhibitors from the most primitive of the Leguminoseae sub-families, Mimosideae, are closer to that of *Peltophorum* and to STI than to the other two inhibitors from the Papilionideae. The cereal inhibitors are significantly closer to those from the Mimosideae than to those from the other two Leguminoseae sub-families. A complete sequence of the *Peltophorum* inhibitor, as well as further sequences from the Caesalpinoideae and the Papilionideae, should help to clarify the evolutionary relationships suggested by the present data.

Table 3. Binary comparisons (% homology) of inhibitors from the STI (Kunitz) family. Homology was calculated for common segments of the sequences that appear in Fig. 1. Abreviations are as in Fig. 1.

	PAPILIONIDEAE			CESALPINOIDEAE			MIMOSIDEAE			GRAMINEAE		
INHIBITOR	WBI	ELI	STI	PAI	AJI1	AJI2	API	AEI	ASI	BASI	WASI	RPI
WBI		66	45	48	40	46	34	37	41	28	26	41
ELI			42	45	33	39	34	33	37	26	26	33
STI				67	51	52	39	43	37	30	30	41
PAI					50	48	52	45	53	33	38	43
AJI1						59	68	79	53	32	32	30
AJI2							68	58	54	37	42	43
API								61	49	26	26	41
AEI									65	32	36	27
ASI										36	36	30
BASI											89	67
WASI												67
RPI												

The reactive bond has been identified in five of the Leguminoseae inhibitors as Arg-Ile or Arg-Ser in exactly homologous positions (Fig. 1). Since no chymotrypsin inhibitor of this group has been sequenced, the apparent conservation of Arg at the P1 position probably reflects this bias. The inhibitors from wheat and barley, which do not inhibit trypsin, respectively have Gly-Ala and Val-Ala in the homologous positions. High variability for the reactive site amino acids have been recognised since early times in proteinase inhibitor research (see Laskowski and Kato, 1980). More recently, this observation has been extended to those amino acids which are in contact with the enzyme, in the case of the ovomucoids (Laskowski et al., 1987), and to a domain of 16 amino acids around the reactive site, in the case of the serpins (Hill and Hastie, 1987). An X-ray crystallographic analysis of the STIa-porcine trypsin complex indicated that STI was in contact with the enzyme at the following positions (see Fig. 1): Asp-Phe (4-5) Asn (16), Pro-Tyr-Arg-Ile-Arg-Phe (65-70), His-Pro (75- 76). Data in Fig.1 are compatible with contact area hypervariability for this group of inhibitors, with the exception of Asn (16), which seems to be fairly conserved, and the already mentioned Arg at the P1 position.

A search for internal repeats was undertaken following the reports of two binding sites for chymotrypsin in STI (Bosterling and Quast, 1981), in WCI2,3 from winged bean (Shibata et al., 1986), and in DE3 from *Erythrina cristagalli* (Joubert and Sharon, 1985). Mostly imperfect short repeats were found and their distribution was not consistent with a straight-forward two-domain structure. Perhaps when inhibitors with a 1:2 stoichiometry are sequenced, a clearer picture will emerge.

A crystallographic study of wheat WASI is in progress (Maeda et al., 1987) and an elucidation of its reactive site with subtilisin, as well as of the contact areas with both subtilisin and endogenous α-amylase would be of great interest. The 3.0 Å electron density map is in agreement with predictions of secondary structure based on the amino acid sequence and indicates a similar structure to that of STI (Maeda, private communication). In this context, a survey of the activity against endogenous α-amylases of the inhibitors from the Leguminoseae would help to clarify whether this activity was either lost during the evolution of the lipid-storing species, acquired during the evolution of species with starchy seeds, or still conserved in at least some members of both branches.

Genetics

Genetic variants of STI, designated a, b, and c, have been identified, after screening the USDA germplasm collection by polyacrylamide gel electrophoresis, and found to be allelic forms encoded at a single locus for which a null allele also exists (see Orf and Hymowitz, 1979). Isolation and characterization of the genetic variants was carried out (Freed and Ryan, 1978; Kim et al., 1985): the most frequent allele, STIa (88.8%), differs from the

second, STIb (10.9%), at 8 sequence positions, whereas the less frequent one, STIc (0.3%), differs at only one position from STIb, which has led to the speculation that differentiation of the first two alleles was quite ancient and had already been completed in *Glycine soja*, the wild progenitor of the cultivated species *Glycine max* (Kim et al., 1985). The STI locus has been assigned to linkage group 9 in G. max, at 16.2±1.5 map units from the acid phosphatase locus (Hildebrand et al., 1980) and at 15.3±0.9 of the leucine aminopeptidase locus (Kiang and Chiang, 1986).

The gene encoding the barley inhibitor has been assigned to barley chromosome 2, after immunochemical analysis with monospecific antibodies of all barley-wheat addition lines (Hejgaard et al., 1984a), and that for the rye gene has been similarly assigned to chromosome 2R (Hejgaard et al., 1984b).

Physiology

Messenger RNA for STI was isolated and translated *in vitro* by Vodkin (1981). A polypeptide was obtained that had higher apparent M_r than that of the inhibitor obtained from mature seeds, M_r 23,200 vs. M_r 21,500. This finding was confirmed by sequencing a cDNA clone which encoded a protein with a putative leader sequence of 25 amino acids preceding the known sequence of STI (Hoffman et al., 1984). The ultrastructural localization of STI in thin sections was carried out by Horisberger and Tacchini (1982), using specific antibodies and protein-A-gold label. In the cotyledons, STI was found in the cell wall and in most of the protein bodies, but not in the cytoplasm, while in the embryonic axis, some of the label was also associated with the cytoplasm. An investigation of the fate of STI during germination has shown a rather precise proteolytic processing at the carboxyl terminus, which seems to involve a five residue sequence in STIa (Hartl et al., 1986). This would argue against its possible role as a store for sulphur amino acids and would suggest some unknown specific function in germination.

The interaction of barley α-amylase II with BASI has been studied in detail by electrophoresis and by a variety of physical methods (Waselake et al., 1983a; Mundy et al., 1983; Halayko et al., 1986). The inhibitor combines with the enzyme at a molar ratio 2:1, affecting an enzymic tryptophan residue which is essential for productive enzyme-substrate binding. The inhibitor seems to be synthesized in barley endosperm at least up to 30 days after anthesis and its mRNA is not detected in mature dry aleurone, although it appears upon "*in vitro*" incubation and is dramatically induced by treatment with abcisic acid (Mundy and Rogers, 1986). In a study where de-embryonated seeds were incubated with labelled amino acids, the enzyme-inhibitor complex was not significantly labelled (Rodaway, 1978), suggesting a possible regulatory role of the inhibitor during seed development, by inhibiting the enzyme during starch synthesis, and/or, in the event of premature sprouting, by preventing starch degradation (Mundy et al., 1983).

BOWMAN-BIRK INHIBITOR FAMILY

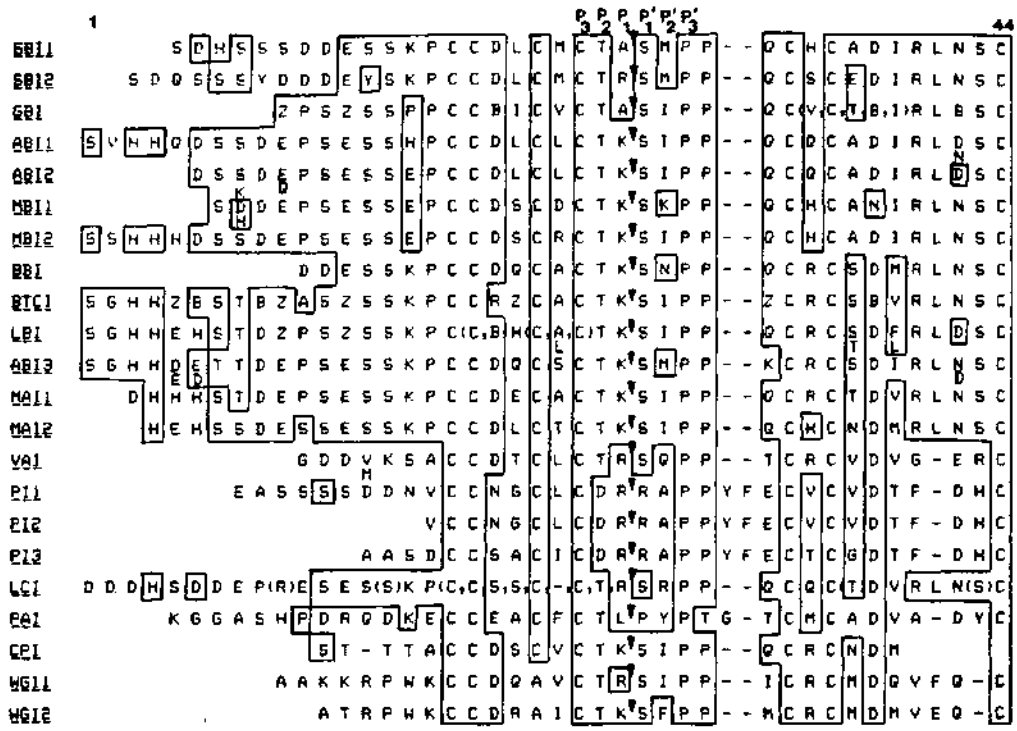
Following the discovery of antitryptic activity in soybeans, two different inhibitors were eventually identified, the already described Kunitz type and a second one, separated by Bowman (1946). A characterization of the second inhibitor was carried out by Birk et al. (1963a,b), hence its present designation as Bowman-Birk inhibitor (BBI), and its covalent structure was established by Odani and Ikenaka (1972, 1973a). Homologues of BBI have been isolated in the seeds of numerous species. A first-hand account of the early research on BBI has been published by Birk (1985) and a thorough review of the BBI family has appeared quite recently (Ikenaka and Norioka, 1986). A succinct description of this family will be presented here and only very recent developments will be treated in some detail.

Distribution and Inhibitory Properties

The Bowman-Birk inhibitor (BBI) is a protein of 71 amino acid residues, with 7 disulphide bridges, that inhibits two serine proteases simultaneously, trypsin and chymotrypsin. The double-headed nature of this inhibitor was elegantly corroborated by Odani and Ikenaka (1973b), who fragmented the molecule with cyanogen bromide and pepsin into the two active domains and a C-terminal tetrapeptide. Homologues of BBI, with two reactive sites and capable of binding two molecules of serine proteases simultaneously, have been described in a number of species. In general, specificity is determined by the residue at P1 position of the reactive site: arginine and lysine for trypsin, tyrosine and phenylalanine for chymotrypsin, and alanine for elastase (see Ikenaka and Norioka, 1986, and Fig. 2). Exceptions are the first reactive sites of the peanut inhibitors (Norioka and Ikenaka, 1983b, 1984) and the second reactive site of the soybean inhibitor C- II (Odani and Ikenaka, 1977), which can bind either trypsin or chymotrypsin, although an Arg residue at the P1 position would predict specificity for trypsin in all the cases. A different exception is that represented by inhibitor II from azuki bean, which can not bind trypsin and chymotrypsin simultaneously, although it is truly double-headed (Yoshikawa and Ogura, 1978).

Inhibitors of the Bowman-Birk type have been described in the seeds of many Leguminoseae: soybean (Odani and Ikenaka, 1972, 1976, 1977), garden bean (Wilson and Laskowski, 1975), azuki bean (Ishikawa et al., 1979; Yoshikawa et al., 1979a; Kiyohara et al., 1981), mung bean (Zhang et al., 1982; Wilson and Chen, 1983), cowpea (Morphy et al., 1985), lima bean (Stevens et al., 1974), *Macrotyloma axillare* (Joubert et al., 1979), *Lonchocarpus capassa* (Joubert, 1984a), *Pterocarpus angolensis* (Joubert, 1982), *Vicia angustifolia* (Shimokawa et al., 1984), peanut (Norioka and Ikenaka, 1983a,b), navy bean (Wagner and Riehm, 1967), kidney bean (Putzai, 1968), black-eyed pea (Gennis and Cantor, 1976), runner bean (Hory and Weder, 1976) and lentil (Weder, 1986). Until recently, the BBI family was thought to

be restricted to the Papilionaceae (Fabaceae), the most advanced sub-family of the Leguminoseae. The recent demonstration by Odani et al. (1986) of significant homology of the germ trypsin inhibitors from cereals with BBI extends the distribution of this type of inhibitor to a distant family and suggests that it may be present in other taxa. Besides the typical two-headed inhibitors, a second single-domain class has been identified in cereals which has not been found in any others species (Odani et al., 1986).



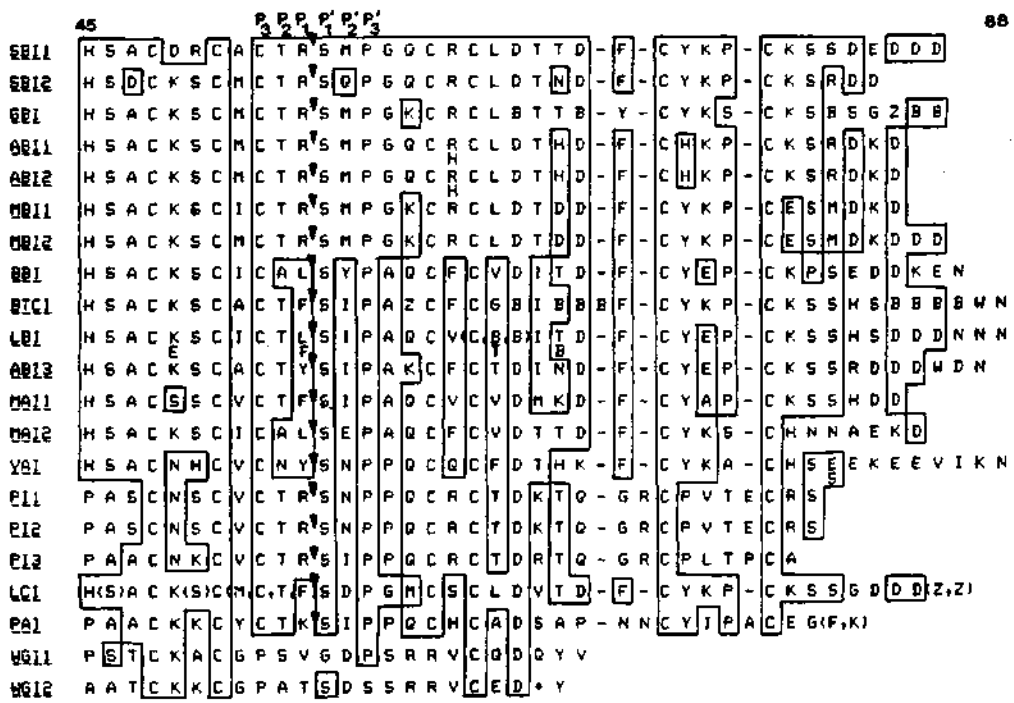


Table 4. Binary comparisons (% homology) of members of the Bowman-Birk inhibitor family. Homology was calculated for common segments of the sequences that appear in Fig.2. Abreviations for inhibitors are as in Fig. 2. Tribes were abreviated as follows: Gly, Glycineae;Pha,Phaseoleae; Vic, Vicieae; Sti,Stilosantheae; Dal, Dalbergiae;Gra, Gramineae.

GROUP →			I							II						III	IV							
↓	INHIBITOR →		SBI1	SBI2	GBI	ABI1	ABI2	MBI1	MBI2	BBI	BTCI	LBI	ABI3	MAI1	MAI2	VAI	PI1	PI2	PI3	LCI	PAI	CPI	WGI1	WGI2
	TRIBE ↓																							
	Gly	SBI1		77	72	73	75	72	75	67	65	63	63	62	64	47	38	35	41	66	39	50	31	31
	Gly	SBI2			69	72	75	69	69	67	59	59	61	60	61	49	38	35	39	63	36	50	35	33
	Pha	GBI				77	78	74	79	57	64	69	65	65	67	44	42	40	41	66	37	61	37	33
I	Pha	ABI1					97	79	83	62	67	69	64	66	65	48	39	35	39	61	36	61	35	35
	Pha	ABI2						82	88	63	69	71	66	68	67	50	41	38	41	65	38	61	35	35
	Pha	MBI1							93	63	67	68	68	67	67	45	35	33	36	64	37	58	31	33
	Pha	MBI2								61	70	69	67	68	68	46	37	32	37	64	37	65	35	35
	Gly	BBI									75	79	75	75	74	45	34	34	36	65	39	61	37	41
	Pha	BTCI										83	76	79	67	44	35	30	37	63	38	61	38	35
	Pha	LBI											77	81	72	44	38	33	41	59	40	65	38	39
II	Pha	ABI3												74	63	43	35	31	37	59	37	58	37	37
	Pha	MAI1													68	48	37	33	39	67	39	65	37	37
	Pha	MAI2														46	35	31	36	58	33	69	35	39
III	Vic	VAI															43	43	43	42	40	54	36	33
	Sti	PI1																87	75	37	37	36	27	27
IV	Sti	PI2																	75	31	34	32	27	27
	Sti	PI3																		37	46	35	29	31
	Dal	LCI																			35	54	33	33
	Dal	PAI																				33	27	31
	Vic	CPI																					54	58
	Gra	WGI1																						66
	Gra	WGI2																						

Figure 2. (opposite). Alignment of amino acid sequences of proteinase inhibitors of the Bowman-Birk family. SBI1 and SBI2 are respectively inhibitors CII and DII from soybean (Odani and Ikenaka, 1976, 1977); GBI is inhibitor II' from garden bean (Wilson and Laskowski, 1975); ABI1 and ABI2 are inhibitors I- A,A' and IIa,c, respectively, from azuki bean (Kiyohara et al., 1981; Ishikawa et al., 1985); MBI1 and MBI2 are two inhibitors from mung bean (Zhang et al., 1982; Wil son and Chen, 1983); BBI is the Bowman-Birk inhibitor from soybean (Odani and Ikenaka, 1972); BTCI is an inhibitor from cowpea (Morphy et al., 1985); LBI is inhibitor IV-IV' from lima bean (Stevens et al., 1974); ABI3 is inhibitor I-II from azuki bean (Ishikawa et al., 1979; Yoshikawa et al., 1979a); MAI1 and MAI2 are respectively inhibitors DE3 and DE4 of Macrotyloma axillare *(Joubert et al., 1979); VAI is an inhibitor from* Vicia angustifolia *(Shimokawa et al. , 1984); PI1, PI2 and PI3 are respectively inhibitors AII, BIII, and BII from peanuts (Norioka and Ikenaka, 1983a,b); LCI is inhibitor DE4 from* Lonchocarpus capassa *(Joubert, 1984a); PAI is inhibitor DE1 from* Pterocarpus angolensis *(Joubert, 1982); CPI is an inhibitor from chickpea (Belew and Eaker, 1976); WGI1 and WGI2 are inhibitors from wheat germ (Odani et al., 1986). Vertical arrows (▼) indicate the reactive bonds. Gaps are indicated (-)*

Structure and Evolution

Inhibitors of the BBI family have been classified into four groups according to their sequence homology and a phylogenetic tree has also been deduced (see Ikenaka and Norioka, 1986). In Fig. 2 the sequences included in the alluded classification have been aligned with the following additional ones: cowpea inhibitor (Morphy et al., 1985), chickpea inhibitor (Belew and Eaker, 1976), two sequences from species of the tribe Dalbergieae, namely *Lonchocarpus capassa* (Joubert, 1984a) and *Pterocarpus angolensis* (Joubert, 1982), and two trypsin inhibitors from wheat germ (Odani et al., 1986). The percentage of homology for the length of sequence compared in each case is presented for all binary comparisons in Table 4. From these data the following aspects merit attention:-

i) Although quite diverged, the cereal inhibitors are more closely related to each other than to any other of the inhibitors.

ii) The inhibitor from chick pea is about equidistant to the cereal inhibitors (54%-58% homology), to group I (50%-65%), to group II (61%-69%), and to the only other sequenced inhibitor from the tribe Vicieae (Group III, 54%).

iii) The inhibitor from cowpea fits into group II.

iv) The two inhibitors from the tribe Dalbergieae are quite different from each other (35% homology); while LCI-DE4 is close and about equidistant to group I (61%-66%) and to group II (58%-67%), PAI-DE1 is quite apart from all the other inhibitors (33%-46%).

v) Inhibitors from the same species, i.e. soybean, belong to more than one group.

These observations clearly indicate that a good part of the divergence of the multigene families encoding inhibitors of the BBI-type occurred before speciation of the Leguminoseae and that the apparent lack of correlation between the sequence data and the taxonomic classification is due to non-homology of the loci being compared, which makes the above-mentioned phylogenetic tree difficult to interpret in evolutive terms. Besides sequencing new inhibitors from new species, a clear priority from an evolutionary point of view would be to determine all the different sequences of BBI-type inhibitors within a number of species and to investigate the genome organization of the corresponding multi-gene families.

An ancestral monovalent inhibitor that would have generated the double-headed ones by internal gene duplication has been postulated (Tan and Stevens, 1971; Odani and Ikenaka, 1976). In this context, the recent finding of single-headed inhibitors in wheat germ further supports the hypothesis and suggests that these are the most primitive among the known members of this family. Then, the cereal double-headed inhibitors would be the closest to the ancestral double-headed inhibitor, being the less diverged from the single-headed one, and the inhibitor from chickpea would be the most primitive among the dicot inhibitors, being the closest to the wheat ones. However, the single-domain cereal inhibitors could be the result of post-translational processing of double-headed precursor or could be encoded by genes originated through deletion of the second

domain sequence. Molecular cloning of the pertinent genes and further protein sequences might allow to discriminate between these alternatives.

Very recently, the structure of the BBI-type inhibitors AII from peanut has been determined by X-ray crystallography at a 3.3 Å resolution (Suzuki et al., 1987). The structures of the two domains are similar and are related by an intramolecular approximate twofold rotation axis, while the two independent protease binding sites protrude from the molecular body on opposite sides. Based on this three-dimensional structure, the evolutionary scheme proposed by Odani and Ikenaka (1976) has been further refined (Suzuki et al., 1987), postulating a second partial duplication, that would have extended the C- terminal and in turn replaced the N-terminal residues of the first domain, and a change of the pairing partners of the disulphide bridges, as represented in Fig. 3.

Other recent structural studies have been carried out with inhibitor BIII from peanut, using 1H NMR (Koyama et al., 1986), and with the complex of bovine trypsin and inhibitor ABI from azuki beans (Tsunogae et al., 1986). The first study indicated that cleavage of the two reactive sites resulted in conformational changes that exclusively affected the disulphide loops containing the reactive sites. From the second work it was concluded that, similarly to other families of inhibitors, both the primary and the secondary contact regions ("front side" and "back side" of the ring) were essential for the inhibitory interaction.

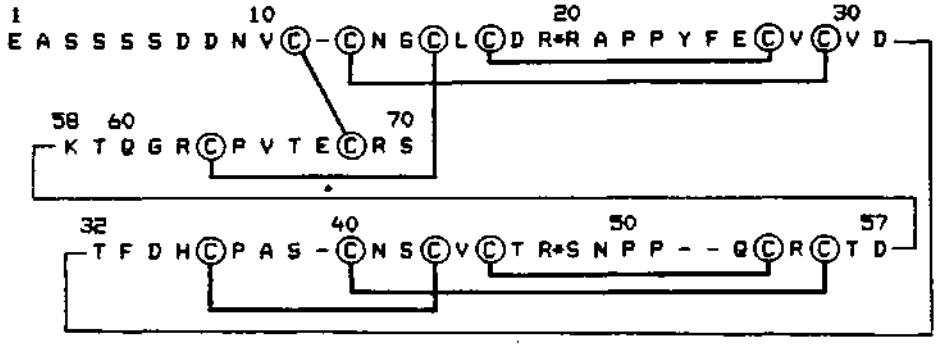


Figure. 3. The primary sequence of PII from peanut, aligned to highlight the three-dimensional domain structure and internal homology, according to Suzuki et al. (1987). The reactive sites are marked by asterisks and thick lines show disulphide bridges.

Genetics and Molecular Cloning

There is a surprising lack of genetic studies concerning the intraspecific variation and genome organization of the multi-gene families encoding the BBI family of inhibitors. As already pointed out, such studies would greatly help our understanding of the evolution of this protein family. The molecular cloning and analysis of a gene coding for BBI has been reported by Larkins and co-workers (Hammond et al., 1984). They constructed and characterized a cDNA clone corresponding to BBI and, using this clone as a probe, determined that the BBI gene was present in only one or two copies per genome. They did not find cDNA clones for other members of the family. Sequence analysis of a genomic clone revealed that it was similar, but not identical, to the cDNA sequence and that it had no introns. Additional cDNA and genomic sequences will have to be analysed to explain the significance of the observed variation between the two clones. It is of interest to note that while the BBI cDNA probe did not hybridize with other genes of the BBI family in soybean, it was able to do so with homologous genes from mimosa and redbud. Based on the DNA sequence, it seems that the initial translation product must contain a short leader sequence. An estimate of divergence time of 310 million years was made for the first and second domains of BBI based on the divergence of their nucleotide sequences.

Physiology

The BBI and its homologues do not appear to accumulate in any plant tissue other than the developing seed (Hwang et al., 1978). BBI mRNA accumulates early during the mid-maturation stage and reaches a steady state later in development (Foard et al., 1982; Hammond et al., 1984). The quantity of protein accumulated is of the same order as that for the Kunitz inhibitor.

During germination, at least part of the inhibitor is rapidly released from the seed within the first 8h of imbibition (Hwang et al., 1978; Wilson, 1980; Tan-Wilson and Wilson, 1982; Horisberger and Tacchini, 1982). The rapid release suggests its elution from a barrier-free pool, which is in line with the fact that BBI is detected in the intercellular space prior to germination but is absent from this space in 4-day old seedlings. Besides this release, it has now been documented in azuki bean (Yoshikawa et al., 1979b), soybean (Madden et al., 1985), and mung bean (Lorensen et al., 1981; Wilson and Chen, 1983) that this type of inhibitor undergoes a specific and extensive proteolytic processing during the early stages of germination and seedling growth. The significance of these phenomena is unclear, but they seem to exclude a reserve role for this protein family.

CEREAL TRYPSIN/α-AMYLASE INHIBITOR FAMILY

This protein family includes a wide range of components whose structural relationships, often unsuspected, have only recently been fully demonstrated. Inhibitors of heterologous α-amylases were first described in wheat endosperm by Kneen and Sandstedt (1943). Years later, work on the main wheat albumins (Fish and Abbot, 1969; Ewart, 1969; Feillet and Nimmo, 1970; Sodini et al., 1970; Cantagalli et al., 1971, and others), which were found to be identical to some of the inhibitors, led to the realization that the inhibitors represent a major part of the albumin and globulin fraction of the endosperm. The first trypsin inhibitor of this family was isolated from barley endosperm by Mikola and Suolinna (1969), but its relationship to the α-amylase inhibitors was discovered only recently (Odani et al., 1982, 1983a). The CM- proteins from wheat, barley, and rye, which were so designated because of their solubility in chloroform:methanol mixtures (Garcia-Olmedo and Garcia-Faure, 1969; Garcia-Olmedo and Carbonero, 1970; Rodriguez-Loperena et al., 1975; Salcedo et al., 1978b, 1982; Paz-Ares et al., 1983a), were eventually found to be members of the trypsin/α-amylase inhibitor family, an observation that led to the discovery of new α-amylase and trypsin inhibitors (Shewry et al., 1984; Barber et al., 1986a,b; Sanchez-Monge et al., 1986b). Finally, homologous relationships of the cereal inhibitors were established with the 2S storage proteins from castor bean (*Ricinus communis*) (Odani et al., 1983c) and the Kazal secretory trypsin inhibitor from bovine pancreas (Odani et al., 1983b), thus showing that this protein super-family is distributed beyond the plant kingdom.

Distribution and Inhibitory Properties

Inhibitors of heterologous α-amylases have been described in a number of species, although the available information for some of them is not as complete as that for those of wheat and barley.

Fractionation of a partially purified NaCl extract by gel filtration allowed a classification of the wheat inhibitors into three classes - monomeric, dimeric and tetrameric - which showed different specificities: the dimeric inhibitor seemed to inhibit mainly α-amylase from human saliva, while all three inhibitors were active against α-amylase from the insect *Tenebrio molitor* (Petrucci et al., 1974; for a review see Buonocore et al., 1977). Purification and characterization of individual inhibitors from wheat was undertaken at several laboratories (Shainkin and Birk, 1970; Saunders and Lang, 1973; Silano et al., 1973; Petrucci et al., 1976, 1978; O'Donnell and McGeeney, 1976; O'Connor and McGeeney, 1981a; Maeda et al., 1982) and two types were characterized, respectively designated 0.28 and 0.19. The first type represented monomeric variants of about M_r 12,000 that were more active against insect α-amylases than against the human salivary or pancreatic ones. Those of the second

type were dimeric of about M_r 24,000 and were more effective against insect enzymes. The dimers could be dissociated into M_r 12,000 subunits that were initially thought to be different (Silano et al., 1973), although more recent evidence suggests that they might be mixtures of homodimers (Maeda et al., 1985). Both types seem to have one molecule of carbohydrate per subunit, which might be important for their inhibitory activity (Silano et al., 1977; Petrucci et al., 1978), and to share a number of physicochemical characteristics (Silano et al., 1973; Silano and Zahnley, 1978). Their interaction with the enzyme was also studied and stoichiometries of 2:1 for 0.28 and 1:1 for 0.19 were proposed. The sugar moieties were suspected to be essential for the formation of the complex, which would explain the reversion of the inhibition by maltose (Silano et al., 1977; and Buonocore et al., 1980).

The wheat tetrameric inhibitor has been less actively investigated. O'Connor and McGeeney (1981a) isolated an inhibitor with an apparent M_r of 63,000, which dissociated into subunits of M_r 14,000 and 15,000 with no carbohydrate, while Buonocore et al. (1985) determined an apparent M_r of 48,000 by equilibrium sedimentation and separated four electrophoretic bands, all of which seemed to be inhibitory. According to these authors, this inhibitor was active against both insect and human α-amylases but not against those from bacteria (*Bacillus subtilis*) or from fungi (*Aspergillus oryzae*). Amino acid analyses and circular dicroism spectra of the subunits indicated that they were similar to those of the monomeric and dimeric inhibitors. Recent reconstitution experiments have shown that proteins CM2, CM3 and CM16 from tetraploid wheat and the same proteins plus CM1 and CM17 from hexaploid wheat are components of the tetrameric inhibitors (Sanchez-Monge, unpublished), thus identifying the subunits of the inhibitors described by Buonocore et al. (1985).

An extensive survey of the inhibitory properties of the three size classes of inhibitors versus α-amylases from 18 insect, 23 marine, and 17 avian and mammalian species was carried out by Silano et al. (1975). It was found that the monomeric class was mostly effective against the insect enzymes, especially against those of grain predators, the M_r 24,000 class was more effective against marine, avian and mammalian α-amylases, and no clear pattern was observed for the tetrameric class. These findings probably reflect the properties of the predominant components within each class, and not the properties of specific purified components. Thus Orlando et al. (1983) found inhibition of the *B.subtilis* α-amylase by components of the 0.19 family, and the monomeric fraction from *Aegilops speltoides* also inhibits the human enzyme (Bedetti et al., 1974).

The existence of α-amylase inhibitors corresponding to the three size classes has been recently demonstrated in barley, and the previously characterized proteins CMa, CMb and CMd have been identified as subunits of the tetrameric inhibitor (Sanchez-Monge et al., 1986b). These three proteins have been shown to be truly homologous to those of the monomeric and dimeric inhibitors from wheat and only one of them, CMa, has been found to be active by itself (Barber et al., 1986a,b; Sanchez-Monge et al., 1986b). Reconstitution experiments indicated that all binary mixtures were

active, including those between inactive subunits, and that the mixture of the three subunits had the highest specific activity (Sanchez-Monge et al., 1986b). The barley tetrameric inhibitor was active against the α-amylase from *T.molitor* but showed no effect against salivary α-amylase. Evidence has also been obtained for the presence of a tetrameric inhibitor with similar subunits in the wild barley *Hordeum chilense* (Sanchez-Monge et al., 1987).

A barley protein designated CMb' has been recently characterized as the subunit of a homodimeric α-amylase inhibitor that seems to be active against α-amylase from *T. molitor* but not against the salivary one (Sanchez-Monge, unpublished).

The only reported inhibitor from this family which has been found to be bifunctional was isolated from ragi (*Eleusine coracana*) by Shivarat and Pattabiranam (1980, 1981). This inhibitor is able to inhibit α-amylase and trypsin independently and is able to form a ternary complex with the two enzymes. The α-amylases of porcine pancreas, human pancreas and human saliva were inhibited in the ratio 5:5:1. A probable isoform of this inhibitor was later isolated by Manjunath et al. (1983).

Oligomeric inhibitors of α-amylase have been identified in *Phaseolus vulgaris* (Pick and Wober, 1978; Lajolo and Finardi- Filho, 1985). There is no evidence that the *Phaseolus* inhibitor is related to the cereal ones, whereas the amino acid composition of that from black bean is quite untypical of this group.

Other α-amylase inhibitors, whose assignment to this family is still uncertain, are heterodimers with the subunits linked by disulphide bridges; they have been found in rye (Granum, 1978), pearl millet (Chandrasekher and Pattabiranam, 1985), sorghum (Moideen Kutty and Pattabiranam, 1986) and *Echinocloa frumentacea* (Moideen Kutty and Pattabiranam, 1985). Finally, a 24,000 M_r heterodimer has been described in *Setaria italica* that is made up of non-covalently linked subunits of M_r 12,000 and 16,000 (Nagaraj and Pattabiranam, 1985). More structural information about these inhibitors will be needed for their proper classification. It is not unlikely that at least some of them might have to be included in a new class.

Besides the trypsin inhibitor from ragi, which is bifunctional, other trypsin inhibitors from this group have been identified in barley, maize, rye, rice, and probably in wheat and sorghum.

The barley inhibitor was first identified by Mikola and Suolinna (1969), who isolated a M_r 14,100 protein from endosperm active against trypsin and inactive against chymotrypsin, papain, subtilopeptidase A, pepsin, bacterial or fungal proteinases, as well as against the endogenous proteinases from green malt. Antibodies raised against this protein did not react with inhibitors from barley embryo or with wheat endosperm extract; cross-reactivity with rye endosperm extract was observed (Mikola and Kirsi, 1972). A similar inhibitor was purified by Boisen (1976) from a different genetic stock. The inhibitor was sequenced by Odani et al. (1983a), who did not find activity against elastase or α-amylases from various sources.

Inhibitors which are probably related to that from barley have been characterized in wheat endosperm (Shyamala and Lyman, 1964; Boisen and Djurtoft, 1981a; Mitsunaga et al., 1982). At least four different species seem to be present in this tissue, which inhibit trypsin at a 1:1 ratio and chymotrypsin in a non-stoichiometric manner. They have similar heat stability and isoelectric points to that of barley, and their reported amino acid compositions are compatible with interspecific homology, although they are moderately divergent.

A trypsin inhibitor which seems to be closely related to that of barley has been characterized in rye (Polanowski, 1974; Boisen and Djurtoff, 1981b; Chang and Tsen, 1981a,b). This inhibitor is also weakly active against chymotrypsin.

The trypsin inhibitor from maize has been well characterized. It was first isolated from the opaque-2 mutant as a M_r 11,000 protein which inhibited trypsin by forming a 1:1 complex and was inactive against chymotrypsin (Swartz et al., 1977). The complete amino acid sequence of this inhibitor was obtained and found to be homologous to the barley one (Mahoney et al., 1984). Similar or identical inhibitors have been identified in different types of maize (Johnson et al., 1980) and in teosinte (Corfman and Reeck, 1982). An of the Hageman factor fragment for which three variants were isolated from maize by Hojima et al. (1980a) is probably identical to the trypsin inhibitor.

An inhibitor from rice bran of about M_r 14,500 which forms a 2:1 complex with trypsin (Tashiro and Maki, 1979; Maki et al., 1980) and one from sorghum (Filho, 1974) seem to be also of the barley type.

No inhibitory properties have been described for the 2S storage proteins from *Ricinus* (Sharief and Li, 1982), *Brassica* (Crouch et al., 1983), *Lupinus* (Lilley and Inglis, 1986) or *Bertholletia* (Ampe et al., 1986).

Structure and Evolution

Total or partial amino acid sequences have been obtained for different members of this protein family either by direct protein sequencing or indirectly from cloned cDNAs (Fig. 4). Percentages of homology calculated for all possible binary comparisons are presented in Table 5. A higher number of members have been characterized in wheat and barley than in any other species. Evolutionary implications of the observed homologies are best understood if they are considered together with the "*in vitro*" activities of the proteins and the chromosomal locations of their corresponding genes in both species. The distribution among chromosomes of the multi-gene family encoding this group of proteins has been investigated through the analysis of wheat aneuploids and barley-wheat addition lines. In the case of wheat and barley, a number of loci can be postulated based on the general observation that there is greater homology between a given

Table 5. Binary comparisons (% homology) of members of the cereal α-amylase/trypsin inhibitor family. Homology was calculated for common segments of the sequences that appear in Fig. 4. Abreviations are as in Fig. 4.

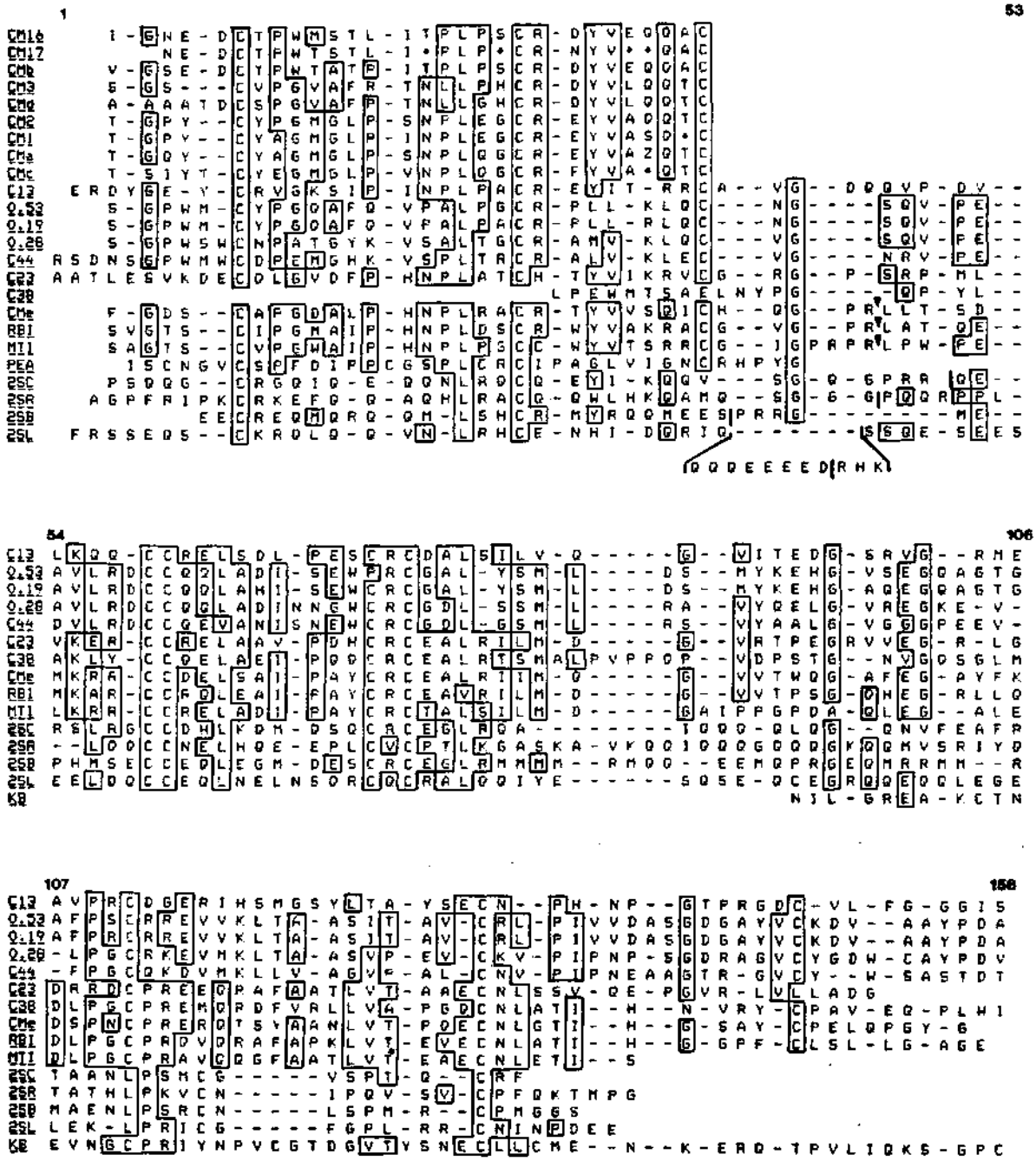
PROTEINS / SPECIES		CM16	CM17	CMb	CM3	CMd	CM2	CM1	CMa	CMc	C13	0.53	0.19	0.28	C44	C23	C38	CMe	RBI	MTI	PA	2SC	2SR	2SB	2SL	KB
WHEAT	CM16		78	83	45	40	45	41	38	33	35	27	27	27	32	22		38	43	37	19	17	16	27	13	
WHEAT	CM17			70	37	36	37	37	35	33	37	26	26	30	30	25		37	37	33	20	19	18	19	19	
BARLEY	CMb				52	47	45	41	38	33	35	30	30	30	29	26		45	47	43	22	17	16	23	13	
WHEAT	CM3					70	50	39	43	38	34	41	41	30	26	32		46	45	48	16	17	13	27	24	
BARLEY	CMd						47	37	40	43	29	30	30	23	22	35		43	39	35	22	16	13	27	19	
WHEAT	CM2							86	86	72	40	38	34	37	39	32		54	52	41	22	21	13	27	17	
WHEAT	CM1								82	69	43	34	31	33	35	32		53	48	41	19	21	13	23	17	
BARLEY	CMa									78	41	31	28	30	32	32		50	48	38	19	24	13	23	21	
BARLEY	CMc										34	31	28	30	32	35		45	43	33	22	17	13	27	20	
BARLEY	C13											22	23	23	22	30	23	36	34	38	19	15	12	16	16	17
WHEAT	0.53												94	58	45	26	27	28	29	30	19	19	15	17	21	11
WHEAT	0.19													56	44	26	28	30	29	30	19	20	16	18	23	11
WHEAT	0.28														63	25	24	25	30	29	22	19	15	17	19	10
BARLEY	C44															24	22	23	26	26	27	17	14	16	16	8
BARLEY	C23																32	42	49	47	22	18	13	19	16	16
BARLEY	C38																	34	35	31	7	16	15	17	16	15
BARLEY	CMe																		56	51	27	23	14	18	18	22
RAGI	RBI																			64	22	19	14	20	18	21
MAIZE	MTI																				22	16	12	15	18	24
PEA	PA																					11	14	12	8	
CA.BEAN	2SC																						35	30	28	8
RAPE	2SR																							21	22	9
BR.NUT	2SB																								22	6
LUPIN	2SL																									14
COW	KB																									

protein from one genome and the appropriate one from a different genome than between that protein and any other encoded in the same genome. The proposed loci are listed together with the *in vitro* activities of the corresponding proteins in Table 6. These data indicate that this dispersed multi-gene family has originated both by translocation and by intrachromosomal duplication, and that most if not all of the dispersion must have occurred prior to the branching-out of the barley genome from the diploid genomes included in allohexaploid wheat. The clearest case of an intrachromosomal duplication is that of the Cma and Cmc loci in chromosome 1 of barley, whose corresponding proteins are closer to each other than to any other encoded in the same genome. Nevertheless, protein CMa shows an even higher similarity to proteins CM1 and CM2, which are respectively encoded in chromosomes 7D and 7B of wheat and, as CMa, are subunits of the tetrameric α-amylase inhibitors. In contrast, CMc is a trypsin inhibitor which shows a much greater divergence from CMe, the other trypsin inhibitor encoded in chromosome 3, than from CMa (45% homology vs 78%).

Figure 4. (opposite) Alignment of amino acid sequences of members of the cereal α-amylase/trypsin inhibitors family. Wheat CM-proteins CM1, CM2, CM3, CM16 and CM17 are subunits of tetrameric α-amylase inhibitors (Shewry et al., 1984; Barber et al., 1986a); barley CM-proteins CMa, CMb and CMd are subunits of tetrameric α-amylase inhibitors (Barber et al., 1986b) and CMc and CMe are trypsin inhibitors (Shewry et al., 1984; Barber et al., 1986a; Odani et al., 1983a; Lazaro et al., 1985); 0.19 and 0.53 are wheat dimeric α-amylase inhibitors (Maeda et al., 1985) and 0.28 is a wheat monomeric α-amylase inhibitor (Kashlan and Richardson, 1981); C13, C23, C38 and C44 are amino acid sequences deduced from barley cDNA clones pUP13, pUP23, pUP38 and pUP44, respectively, the latter corresponds to a dimeric α-amylase inhibitor (Paz-Ares et al., 1986; Lazaro, unpublished); RBI is an α-amylase/trypsin inhibitor from ragi, Eleusine coracana *(Campos and Richardson, 1983); MTI is a trypsin inhibitor from maize (Mahoney et al., 1984); PA is a sulphur-rich pea albumin (Higgins et al., 1986); 2SC, 2SR, 2SB and 2SL are 2S storage proteins from* Ricinus communis *(Sharief and Li, 1982),* Brassica napus *(Crouch et al., 1983),* Bertholletia excelsa *(Ampe et al., 1986), and* Lupinus angustigolius *(Lilley and Inglis, 1986), respectively; KB is the bovine Kazal inhibitor (Greene and B artelt, 1969). Gaps introduced for the alignment are indicated (-). Vertical arrows (▼) indicate the reactive bonds of the trypsin inhibitors. The three sequence blocks approximately correspond to the three domains defined by Kreis et al. (1985). The N-terminal positions of the second chain of the two chained 2S globulins are indicated by a vertical line (|). Conserved positions are boxed. Unidentified residues are indicated by an asterisk (*).*

Over a dozen different sequences of this family have been detected as abundant proteins and/or mRNAs in the endosperm of barley (Salcedo et al., 1984; Paz-Ares et al., 1986 and unpublished), which is a diploid species, whereas the number of variants per genome in wheat seems to be lower, possibly as a result of diploidization (Aragoncillo et al., 1975; Sanchez-Monge et al., 1986a).

Because of the complexity of this protein family, as has been ascertained in wheat and barley, the evolutionary implications of the limited sequence information available in other species must be drawn with caution. Thus, the bifunctional inhibitor from *E. coracana* has the highest homology with the maize trypsin inhibitor, followed by barley trypsin inhibitor CMe, the barley protein encoded by cDNA clone pUP-23, and some of the wheat and barley subunits of the tetrameric α-amylase inhibitors, but proteins with higher percentage of interspecific homology may yet be found in these species (Table 5). Protein PA from pea seeds is quite distant from the wheat and barley proteins, showing the highest homology with barley trypsin inhibitor CMe and with the barley protein encoded by clone pUP44. It should be pointed out that in this and subsequent cases of weak homology (20%-30% range), most of the homology is due to highly conserved or invariant positions, most notably the cysteines (Fig. 4).



There is considerable divergence within the group of 2S storage proteins, which have no known inhibitory activity, and between this group and the rest of the sequenced proteins. The intra-group homology is in the 20%-35% range, whereas homology with the nearest cereal components is in the 18%-27% range (Table 5).

Table 6. Chromosomal locations of genes and inhibitory activities of the α-amylase/trypsin inhibitors from wheat and barley.

Chromosome group	Genome*	Species	Protein grouped by loci	Inhibitory activity	References**
3	B	W	I (0.53)		a,b,c
	D	W	III (0.19)	α-amylase (dimeric)	a,b,c
?	H	B	CMb' (c44)		d
	H	B	CMe	trypsin	e,f
4	A	W	CM16		a,b
	D	W	CM17	α-amylase (tetrameric)	a,b
	H	B	CMb		e
	A	W	CM3		a,b
	H	B	CMd	α-amylase (tetrameric)	e
6	D	W	II (0.28)	α-amylase (monomeric)	b,c,g
7	B	W	CM2		a,b,h
	D	W	CM1	α-amylase (tetrameric)	a,b,h
1*	H	B	CMa		e
	H	B	CMc	trypsin	e
?	H	B	C13,C23,C38	?	i,j

* Hexaploid wheat (T. aestivum): genomes AABBDD and barley (H. vulgare): genomes HH. Barley chromosome 1 homologous to chromosome group 7 of wheat.

** a) Aragoncillo et al. (1975); b) Fra-Mon et al. (1984); c) Sanchez-Monge et al. (1986a); d) Sánchez-Monge and Lazaro (unpublished); e) Salcedo et al. (1984); f) Hejgaard et al. (1984); g) Pace et al. (1978); h) García-Olmedo and Carbonero (1970); i) Paz-Ares et al. (1986); j) Lázaro (unpublished).

The Kazal secretory trypsin inhibitor from bovine pancreas shows significant homology only with the trypsin inhibitors from maize, ragi and barley CMe (Table 5). The three domains proposed for barley trypsin inhibitor CMe by Kreis et al. (1985) are approximately indicated in Fig. 4. The reactive site is just at the right-hand border of the first domain and the sequence -Pro-Arg-Leu-, which corresponds to the -P2-P1-P'1- residues, has been conserved in the three proteins with anti-trypsin activity which have been completely sequenced (Fig. 4). Otherwise, the region around the trypsin reactive site appears as extremely variable throughout this family, with numerous deletions and/or insertions.

A weak but significant homology has been found between the alluded domains of these proteins and similar domains present in prolamins such as α-gliadin, B1-hordein, "γ"-secalin, HMW-prolamin, and other cereal prolamins, in which these domains are separated by stretches of repeated sequences (Kreis et al., 1985). The sequence information accumulated since that finding further supports the proposed homology as better fitting comparisons can be made.

The sequences in Fig. 4 were searched for internal repeats because the homology of the Kazal inhibitors with the C- terminal part of these proteins, and the double-headed nature of the bifunctional inhibitor from ragi and of the trypsin inhibitor from rice bran, suggested a possible duplication. Only in the case of the monomeric α-amylase inhibitor (0.28) a weak homology was found between the N-terminal and the C- terminal domains:

```
18
L T G C R A M V   K L Q C V G S Q V P E
.   . . .   .   . .       .   . . .  11/20
L P G C R K E V M K L T A A   S   V P E
                                    96
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Genetics

Apart from the studies already discussed regarding the chromosomal locations of genes encoding proteins of this group, research on other aspects of their genetic control and regulation has also been carried out. The considerable sequence divergence found for this protein family within a genome is in sharp contrast with the low variability which has been observed for the different members of the group. In a survey of tetraploid and hexaploid wheat cultivars, no variants were found for proteins CM1 and CM2, and only a rare allelic variant of protein CM3 was present in a small group of closely related cultivars (Garcia-Olmedo and Garcia-Faure, 1969; Garcia-Olmedo and Carbonero, 1970; Rodriguez-Loperena et al., 1975; Salcedo et al., 1978a). Similarly, in a wide-ranging survey of *Hordeum vulgare* cultivars and *H. spontaneum* accessions, proteins CMa and CMc were invariant in the cultivated species and presented rare variants in the wild one, protein CMd was invariant in both species, and proteins CMb and CMe had allelic variants in both species (Salcedo et al., 1984; Molina-Cano et al., 1987). In the case of the trypsin inhibitor CMe, two variants, designated CMe2/CMe2', appeared in over 40% of the samples and were jointly inherited and codominantly expressed with respect to CMe (Salcedo et al., 1984).

Other surveys have been concerned with the variation of the inhibitory activity against trypsin (Kirsi, 1973; Kirsi and Ahokas, 1983) and against α-amylases (Bedetti et al., 1974). The considerable variation in the levels of activity indicated that these were probably due not only to changes of the specific activity among variants but also to extreme variations in the expression levels of the corresponding genes.

The regulation of the expression of genes from this family has been studied in some detail in a number of cases. Salcedo et al. (1978a) demonstrated that the net accumulation of protein CM3, now known to be a subunit of the tetrameric α-amylase inhibitor in wheat, and that of its allelic variant CM3', varied linearly with gene dosage but the amount of CM3' for a given dosage was about half of that of CM3. The expression level cosegregated with the structural variation indicating that it was dependent on cis-acting genetic elements. A gene-dosage compensation effect was described by Aragoncillo et al. (1978) for proteins CM16 and CM17, which are also equivalent subunits of the tetrameric α-amylase inhibitor in wheat. Although the net output of each of the proteins varied linearly with gene dosage, the amount of protein at a given dosage of its structural gene was significantly higher if the chromosome carrying the gene for the other protein was absent. This suggests some degree of coordinated expression mediated by trans-acting genetic elements of the genes encoding subunits of the tetramers.

The effects of high-lysine mutations on the expression levels of different genes from this family have been investigated (Salcedo et al., 1984; Lazaro et al., 1985). The most notable effect concerns the gene for the trypsin inhibitor CMe, whose product is not detected in mutant Riso 1508 and is at a much lower level in Hiproly, as has been confirmed recently (Salcedo, unpublished). In both cases, the mutations affect loci in chromosome 7, while the structural gene for the inhibitor is in chromosome 3 (see Lazaro et al., 1985). Just the opposite effect has been described for the high-lysine mutation opaque-2 of maize, which greatly increases the corresponding trypsin inhibitor (Halim et al., 1973).

Physiology

Tissue localization of the barley trypsin inhibitor(s) was found to be restricted to the starchy endosperm and the aleurone layer, as they were not detected in husks, coleoptiles, leaves or roots (Kirsi and Mikola, 1971). Similarly, Buonocore et al. (1977) concluded that the α-amylase inhibitors were endosperm-specific. Synthesis of this protein family seems to precede that of the bulk of reserve proteins and starch. Thus, Kirsi (1973) detected the barley trypsin inhibitor at about 5 days after anthesis (daa) and found that most of it was accumulated between 10 and 23 daa. He did not find a response to nitrogen fertilization. The α-amylase inhibitors were detected at 8 daa (Pace et al., 1978). In a study of in vivo and in vitro synthesis of these proteins in barley, Paz-Ares et al. (1983b) concluded that synthesis took place between 10 and 30 daa, with a peak between 15 and 20 daa. These proteins were synthesized by membrane-bound polysomes as precursors of higher apparent M_r (13,000-21,000) than the mature proteins (12,000-16,000). The largest in vitro product (21,000) was found to be the precursor of subunit CMd. Accordingly, a putative leader sequence has been found in those cDNA clones of this family that were large enough to include the 5'-end of the mRNA sequence (Paz-Ares et al., 1986).

The subcellular location of the different members of this family has not been investigated. Indirect evidence of association with starch granules has been reported (see Buonocore et al., 1977) and indeed at least some of the inhibitors show affinity for insoluble starch (O'Connor and McGeeney, 1981b). However, Paz-Ares et al. (1983b) following different homogenization and subcellular fractionation procedures consistently found these proteins in the supernatant, indicating that their association with the particulate fraction was labile if it existed at all. On the other hand, Zawistowska and Bushuk (1986) recovered CM-proteins in gluten preparations, where they seemed to have a considerable amount of bound polar lipids.

During germination, the anti-trypsin activity disappears rather abruptly after remaining stable for 2-3 days (Kirsi and Mikola, 1971). Similarly, rapid disappearance with the onset of germination have been observed for the α-amylase inhibitor (Pace et al., 1978). These observations suggest that these inhibitors do not play a specific role during germination.

POTATO INHIBITORS I AND II FAMILIES

The families of the non-homologous potato inhibitors I and II will be considered together because the two inhibitors play a joint role in the systemic response to wounding (see Ryan, 1984). They were first discovered in potato tubers, where they accumulate throughout development and represent a substantial fraction of the soluble protein, and later they were found to accumulate in wounded tomato and potato leaves (Green and Ryan, 1972; Plunkett et al., 1982; Sanchez-Serrano et al., 1986; Cleveland et al., 1987).

Distribution and Inhibitory Properties

Inhibitor I is an oligomer of M_r 41,000, made of protomers of M_r 8,100, with one disulphide bond and one reactive site each, which inhibits chymotrypsin-like enzymes (Ryan, 1984). Besides potato, tomato and other Solanaceae (Gurusiddaiah et al., 1972; Lee et al., 1986), homologues of this inhibitor have been found in plant species outside this family, such as broad bean (Svendsen et al., 1984) and barley (Svendsen et al., 1980, 1982; Jonassen and Svendsen, 1982), and in a lower animal, the leech (Seemuller et al., 1980).

The inhibitor from broad bean (*Vicia faba*) has no cysteine and is active against microbial serine proteases, including subtilisin, with which it forms a 1:1 complex, and is inactive against chymotrypsin and trypsin (Svendsen et al., 1984). The subtilisin inhibitors from *Vigna unguiculata* and *Phaseolus vulgaris* seem to be closely related

to that from *Vicia faba* as judged from the amino acid compositions (Vartak et al., 1980; Seidl et al., 1978, 1982). It is very probable that the constitutive trypsin inhibitors from buckwheat, reported by Kiyohara and Iwasaki (1985), are also members of this protein family.

Two inhibitors, CI-1 and CI-2, have been well characterized in barley. Both of them inhibit chymotrypsin and subtilisin, but CI-1 has a single reactive site for the two enzymes and CI- 2 has one reactive site for chymotrypsin and two for subtilisin (Jonassen and Svendsen, 1982). Neither of the inhibitors has cysteines.

Eglin, the inhibitor from the leech, has no cysteines and inhibits elastase and cathepsin G (Seemuller et al., 1980). A more distantly related inhibitor of this family has been identified in yeast (Maier et al., 1979).

Inhibitor II is a dimer of M_r 23,000, made of two protomers of M_r 12,000, with five disulphide bonds and two reactive sites per monomer, which respectively inhibit chymotrypsin and trypsin (Ryan, 1984). The inhibitors from this family in tomato and potato are related to two smaller trypsin and chymotrypsin inhibitors from potato tubers, PCI-I and PTI-I (Hass et al., 1982) and with another proteinase inhibitor from egg plant (Richardson, 1979). Wound-inducible antitrypsin activity has also been detected in species such as alfalfa, tobacco, strawberry, cucumber, squash, clover, broadbean and grape (Walker-Simmons and Ryan, 1977). It is yet unknown if these inhibitors are homologues of potato inhibitors I or II and in some cases, such as that of the alfalfa inhibitors (Brown and Ryan, 1984), it is suspected that they might belong to entirely different groups.

Structure and Evolution

Available sequence information of the potato inhibitor I family is summarized in Fig. 5. In potatoes, a mixture of 10 or more isoinhibitors are present and variation is observed at 16 out of the 84 positions of the mature protein (Richardson, 1974; Richardson and Cossins, 1974; Graham et al., 1985a,b, Cleveland et al., 1987). The tomato sequence presented 80% homology with respect to the potato isoinhibitors (Fig. 5). As expected, lower homologies were found between the tomato inhibitor and those from barley (CI-1C, 25%; CI-2, 33%) and broad bean (35%). A remarkably low divergence (35% homology) was found for the animal inhibitor Eglin, whereas the homology of the yeast inhibitor (≈16%) was rather weak (Fig. 5). The reactive sites seem to be located at exactly homologous positions (Richardson et al., 1977), except for CI-1C from barley, which has a second subtilisin site (Met30-Ser31) besides the standard one (Jonassen and Svendsen, 1982). In other inhibitor families, the three-dimensional structure is stabilized by disulphide bridges, which seem to be essential for their activity and, accordingly, they are highly conserved throughout evolution. Members of potato

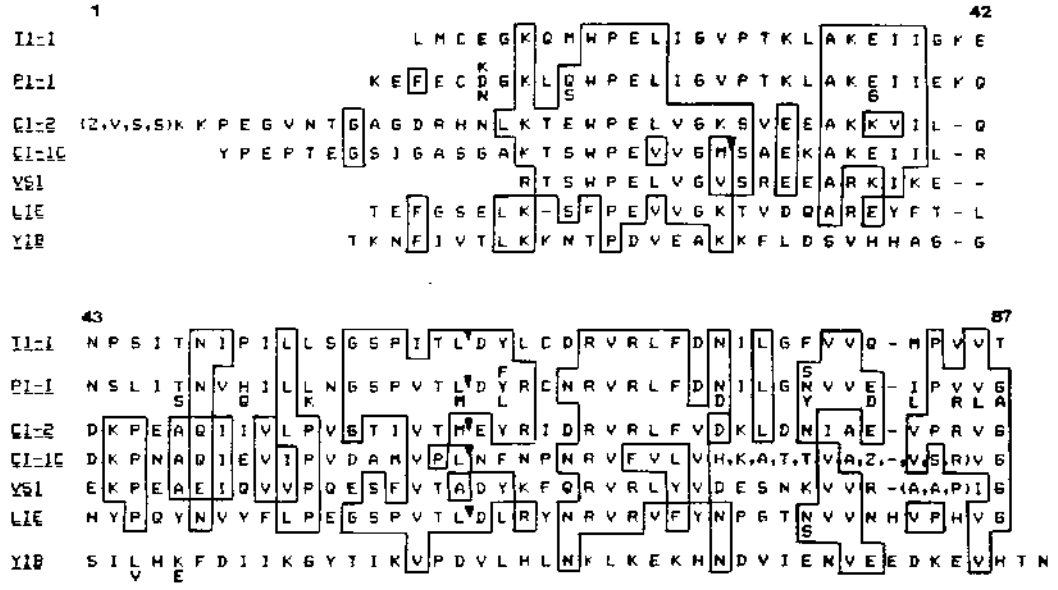

Figure. 5. Alignment of amino acid sequences of members of the potato inhibitor I family. TI-I is from tomatoes (Graham et al., 1985a); PI-I is from potatoes (Melville and Ryan, 1972; Cleveland et al., 1987); CI-2 and CI-1C are from barley (Svendsen et al., 1982); VSI is from broadbean (Svendsen et al.,1984); LIE is the inhibitor Eglin from leech (Seemuller et al., 1980); YIB is from yeast (Maier et al., 1979). Vertical arrows (▼) indicate the reactive bonds. Conserved positions are boxed.

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inhibitor I family either have one disulphide bond or none and it does not seem to be essential for their activity. Indeed, the crystal and molecular structure of barley inhibitor CI-2, which lacks cysteine, and of its complex with subtilisin Novo have been determined and few conformational changes have been found between the free and the complexed inhibitor (McPhalen et al., 1985 and McPhalen and James, 1987).

Although members of the inhibitor II family have been well characterized (Plunkett et al., 1982), direct sequencing methods have not allowed the determination of complete amino acid sequences, which have now been deduced from the corresponding cDNA clones in tomato (Graham et al., 1985b) and in potato (Sanchez-Serrano et al., 1986). Homology between the deduced sequences of tomato and potato is high in the region between positions 29 and 154, where only 19 differences appear (Fig. 6). The preceding signal sequence is 6 amino acids shorter (positions 21 to 28) in the tomato. As shown in Fig. 6, a high degree of homology was observed between the putative inhibitor II from potato, the two smaller proteinase inhibitor peptides from potato tuber, PCI-I and PTI, and a similar inhibitor from eggplant (Richardson, 1979; Hass et al., 1982; Sanchez-Serrano et al., 1986). These smaller peptides could be derived from different isoforms of

inhibitor II by further processing, as suggested by the fact that PCI-I is completely homologous to the appropriate segment of one of the two sequences deduced from potato cDNA (Sanchez-Serrano et al., 1986). As pointed out by Graham et al. (1985b), the full- length inhibitors have a duplicated-domain structure (Fig. 6). Based on the known position of the single reactive site of the eggplant inhibitor (Richardson, 1979), the putative reactive sites of the larger inhibitors have been postulated at the homologous positions of the two domains (Fig. 6), which would explain the known specificities of the different inhibitors (Graham et al., 1985b).

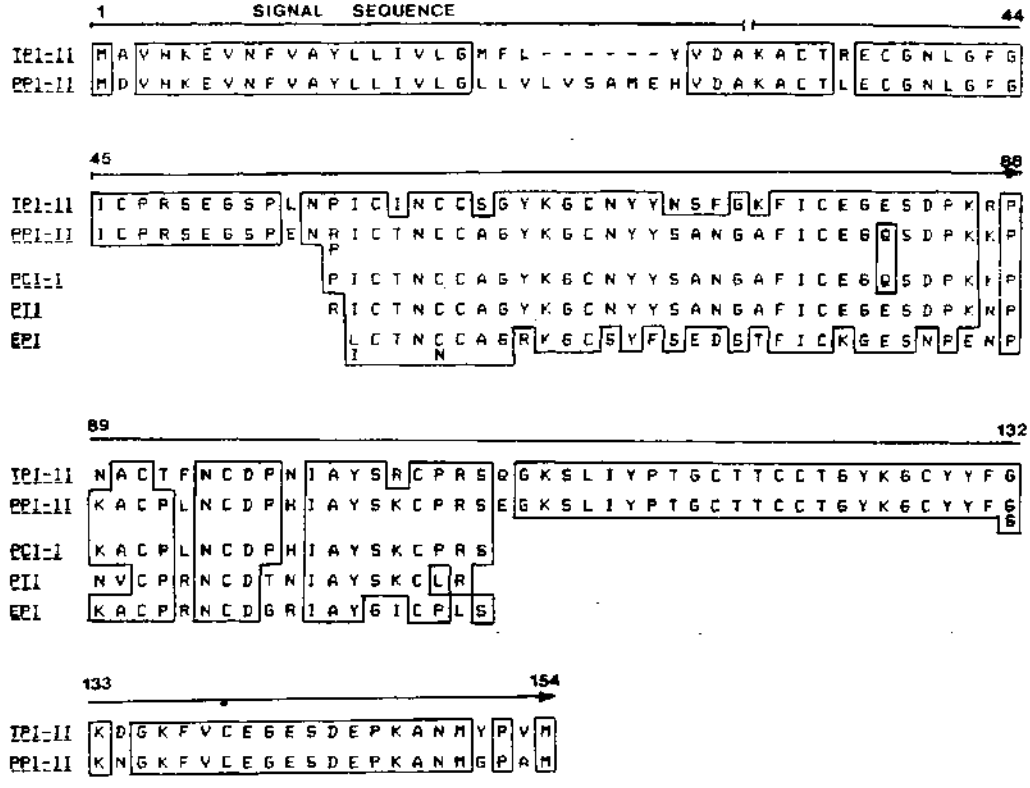

Figure. 6. Alignment of amino acid sequences of members of the potato inhibitor II family. TPI-II is from tomatoes (Graham et al., 1985b); PPI-II is from potatoes (Sanchez-Serrano et al., 1986); PCI-I and PTI are from potatoes (Hass et al., 1982); EPI is from eggplant (Richardson, 1979). Vertical arrows (▼) indicate reactive bonds. Horizontal arrows (►) indicate internal duplications. Conserved positions are boxed.

Genetics and Molecular Cloning

The chromosomal location of genes encoding the chymotrypsin inhibitors in chromosome 5 of barley and the 20-fold increase in their expression as a result of the high-lysine mutation in the locus lys of chromosome 7 have been reported (Jonassen, 1980; Boisen et al., 1981; Hejgaard et al., 1984a). As already discussed, the same high-lysine mutation exerts the opposite effect on the gene encoding barley trypsin inhibitor CMe (Lazaro et al., 1985). The gene for an inhibitor from rye which cross-reacts with antibodies raised against the CI inhibitors from barley has been located in chromosome 1R (Hejgaard et al., 1984b).

Although the variability of the concentration of the inhibitors in potato tubers has been investigated (Ryan et al., 1977), no other formal genetic studies seem to have been carried out concerning these two inhibitor families. In contrast, considerable progress in the molecular cloning of these genes has been achieved in different species: cDNA clones for type-I inhibitors from tomato (Graham et al., 1985a) and barley (Williamson et al., 1987) and for type-II inhibitors from tomato (Graham et al., 1985b) and potato (Sanchez-Serrano et al., 1986) are now available, together with genomic clones for type-I from tomato (Lee et al., 1986) and potato (Cleveland et al., 1987) and for inhibitor-II from potato (Keil et al., 1986).

Comparison of the amino acid sequences, deduced from nucleotide sequences, with the N-terminal sequences of the native inhibitor-I proteins from tomato and potato indicates that in both cases a precursor is coded which must yield the mature protein after processing an N-terminal pre- and pro- sequences. The putative pre- or transit sequences are 23 residues long in both cases, whereas the pro-sequences would have 19 residues, 9 of which are charged in tomato and 16 residues, 6 of which are charged, in potato (Graham et al., 1985a,b, Cleveland et al., 1987). The nucleotide sequences of several cDNAs corresponding to inhibitor CI-2 from barley have an open reading frame that corresponds exactly to the mature protein sequence. Although there is another ATG codon further 69 nucleotides upstream, the sequence between the two ATG codons would code for a typical signal peptide. An in-frame TAA stop codon is consistently present and in vitro translation starts in the downstream ATG (Williamson et al., 1987). The open reading frames in cDNAs corresponding to inhibitor-II in tomato and potato code for precursors of 148 and 154 amino acids, respectively. The N-terminal sequences of these precursors have all the features of typical leader sequences. The tomato leader sequence is six amino acids shorter, which accounts for the difference in length of the precursors (Graham et al., 1985b; Sanchez-Serrano et al., 1986).

Inhibitor-I genes have two introns both in tomato and in potato, whereas a potato gene for inhibitor II has only one intron (Lee et al., 1986; Cleveland et al., 1987; Keil et al., 1986). The cDNA and genomic clones of the inhibitors have been searched for common features that could be related to the regulation of the systemic

wound response. The two tomato proteinase inhibitor mRNAs share a variant polyadenylation signal (AATAAG) and a conserved 3'-untranslated 10-base palindromic region, but these are not present in the potato mRNAs (Graham et al., 1985a,b; Cleveland et al., 1987; Sanchez- Serrano et al., 1986). An imperfect direct repeat 100 bp long was found in the 5'-flanking region of the inhibitor I gene from tomato that was shared by other *Lycopersicon* species with inducible inhibitors, but not by the potato genes for inhibitors I and II (Lee et al., 1986; Cleveland et al., 1987; Keil et al., 1986). Homology of the 3'-untranslated regions of the proteinase inhibitor II genes from potato and an extensin gene have been also pointed out by Sanchez-Serrano et al. (1987), but again this homology is not shared by all the inducible genes.

Physiology

Certain species such as tomatoes, respond to wounding by insects or other severe mechanical damage within a few hours by accumulating proteinase inhibitors in leaves throughout the plant, including non-wounded parts (Green and Ryan, 1972; Plunkett et al., 1982;

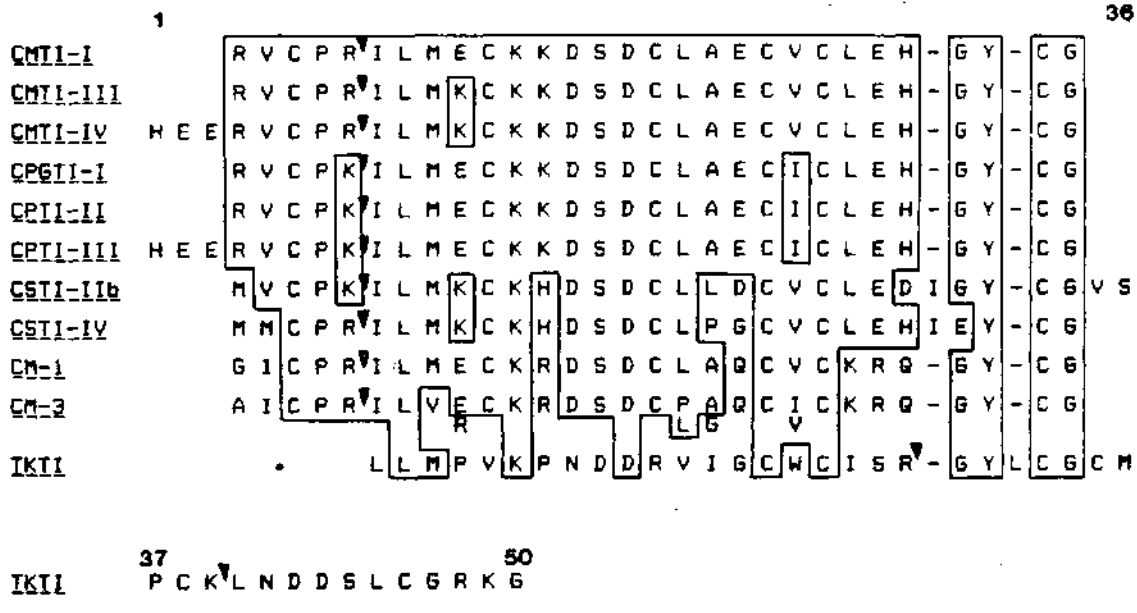

Figure. 7. Alignment of amino acid sequences of the squash family of serine proteinase inhibitors. CMTI-I, CMTI-III and CMTI-IV are from Cucurbita maxima*; CPGTI-I, CPTI-II and CPTI-III are from* C. pepo*; CSTI-IIb and CSTI-IV are from* Cucumis sativus *(Wieczorek et al., 1985). Inhibitors CM-1 and CM-3 are from* Momordica repens *(Joubert, 1984b) and TKTI is from* Trichosanthes kirilowii *(Tan et al., 1984). Conserved positions are boxed. Vertical arrows (▼) indicate the reactive bonds.*

Graham et al., 1986). The induction of the inhibitor proteins is mediated by a putative wound signal, called the proteinase inhibitor inducing factor (PIIF), whose activity has been shown to be associated with fragments of the plant cell wall, apparently released at the wound site by endogenous endopolygalacturonases (Bishop et al., 1984; Ryan, 1984). Following a single wound on a lower leaf of a tomato plant, mRNAs for the two inhibitors began to accumulate in leaves 2 to 4 hours later, and the proteins were detected with a further 2 hours lag. The levels of the mRNAs reached a maximum at about 8 hours after wounding and then decayed with apparent half-lifes of 10 hours; consecutive wounds had an accumulative effect. Synthesis of the inhibitors was found to be regulated at the level of transcription (Graham et al., 1986).

The wound-induced response has been investigated in transgenic tobacco plants in which either the complete inhibitor-II gene from potato (Sanchez-Serrano et al., 1987) or a fusion of this gene with that of chloramphenicol acetyl transferase (Thornburg et al., 1987) had been introduced. The fact that these genes were wound-induced in the transgenic plants indicated that tobacco has the capacity to regulate the expression of these inducible genes. The sequences necessary and sufficient for wound-inducibility were located within 1kbp upstream of the genes.

SQUASH TRYPSIN/HAGEMAN FACTOR INHIBITOR FAMILY

A new family of very small inhibitor (29-32 residues, 3 disulphide bridges) that are active against trypsin and Hageman factor have been described in the seeds of the Cucurbitaceae (Hojima et al., 1980b; Polanowski et al., 1980; Joubert, 1984b). Members of this family have been isolated and sequenced from squash (*Cucurbita maxima*) by Wilusz et al. (1983), from zucchini (*C. pepo* var. Giromontia), summer squash (*C. pepo*), and cucumber (*Cucumis sativus*) by Wieczorek et al. (1985), and from *Momordica repens* by Joubert (1984b). Families of isoinhibitors have been found in all of these species, which seem to result both from multiple structural genes and from proteolytic processing: while some of the sequences are clearly different from each other, in other cases the heterogeneity is only due to the presence or absence of N-terminal peptides (Fig. 7). A double-headed inhibitor from a Chinese medical herb, *Trichosanthes kirilowii* (Cucurbitaceae), which has 41 amino acid residues (Tan et al., 1984), is over 30% homologous to the shorter squash inhibitors and 26% homologous to soybean Bowman-Birk family.

Disulphide bond positions have been proposed, based on the similarity of the squash inhibitors with wheat-germ agglutinin (Siemion et al., 1984). The reactive site is either Arg-Ile or Lys-Ile in agreement with their trypsin specificity (Joubert, 1984b; Wieczorek et al., 1985). In spite of their small size, they are quite strong inhibitors. Up to now these inhibitors have been found only

within the Cucurbitaceae and it will be of interest to investigate their range of distribution and variation.

HOMOLOGY OF PROTEIN Z WITH α1-ANTITRYPSIN AND RELATED PROTEINS

Protein Z is a major barley endosperm albumin of M_r 43,000 which is present in both free and bound forms in the mature grain, where it seems to act as a storage protein and contains a substantial part of the grain lysine (Hejgaard, 1976, 1982; Hejgaard and Boisen, 1980; Giese and Hejgaard, 1984). Nucleotide and amino acid sequencing of cDNA clones have established the 180 C-terminal residues of this protein (Fig. 8). This sequence has been found to be 26-32% homologous with human α1-antitrypsin, human α1-antichymotrypsin, human antithrombin III, mouse contrapsin and chicken ovalbumin (Hejgaard et al., 1985). The sequence homology and the specific cleavage by trypsin at a bond corresponding to the reactive sites of the inhibitors suggest a possible inhibitory activity for protein Z, although no activity against microbial or pancreatic serine proteinases has been found (Hejgaard et al., 1985).

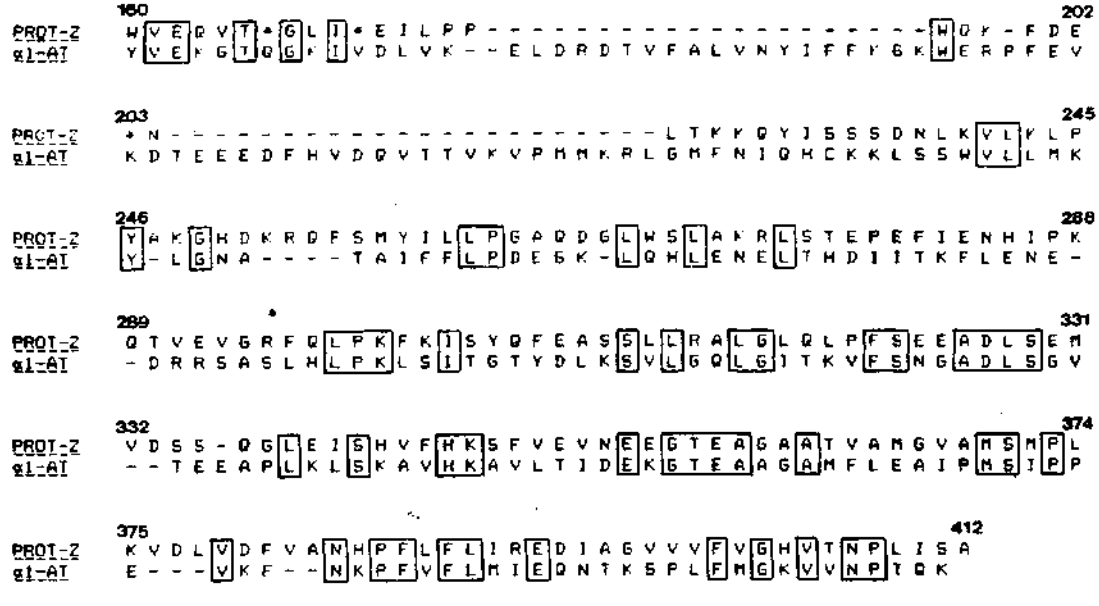

Figure. 8. Comparison of a partial sequence from barley protein Z with α1-antitrypsin (Hejgaard et al., 1985). Conserved positions are boxed.

RAGI I-2 / MAIZE BIFUNCTIONAL INHIBITOR FAMILY

Besides the already described bifunctional inhibitor from ragi (*Eleusine coracana*), a second unrelated inhibitor which was active against porcine pancreatic α-amylase, was characterized in that species (Shivaraj and Pattabiraman, 1980; Campos and Richardson, 1984). A M_r 10,000 protein was also purified from barley and found to be homologous to the ragi inhibitor I-2 (Svensson et al., 1986; Mundy and Rogers, 1986). This protein was considered as a probable amylase/protease inhibitor (PAPI) both because of this relationship with the ragi inhibitor and because of its weak but significant homology with Bowman-Birk type proteinase inhibitors (Mundy and Rogers, 1986), although no target enzyme was identified for it (Svensson et al., 1986). Expression of the gene encoding PAPI, which has been studied using a cDNA probe, takes place primarily in aleurone tissue during late stages of grain development. PAPI mRNA is present at high levels in aleurone tissue of dessicated grain, as well as in incubated aleurone layers prepared from rehydrated grain, PAPI protein being secreted into the incubation medium (Mundy and Rogers, 1986).

The sequence of a maize protein which is a potent in vitro inhibitor of bovine trypsin and insect α-amylase has been recently determined and found to be partially homologous with barley PAPI and ragi I-2 inhibitor (Richardson et al., 1987). The new maize bifunctional inhibitor showed extensive homology with the sweet protein thaumatin II, present in the fruits of *Thaumatococcus danielli*, and with a pathogenesis-related (PR) protein induced in tobacco plants following infection with tobacco mosaic virus. This striking observation suggest a possible inhibitory function both for thaumatin II and the PR- protein (Richardson et al., 1987). Sequence alignments showing the above described relationships are presented in Fig. 9.

INHIBITORS OF METALLOCARBOXYPEPTIDASES

Inhibitors of metallo-carboxypeptidases (CPI) were identified both in potato tubers (Rancour and Ryan, 1968) and tomato fruits (Hass and Ryan, 1980a), and were later found to accumulate in wounded leaves (Graham and Ryan, 1981). These inhibitors are polypeptides of M_r 4,200 which are unusually heat stable. They are active against most animal carboxypeptidases and inactive against all plant and most microbial carboxypeptidases tested (Hass et al., 1981). Inhibition occurs by a competitive mechanism and results in the rapid removal of the carboxy-terminal amino acid, whose cleavage does not lead to elimination of the inhibitory activity (Hass and Ryan, 1980b).

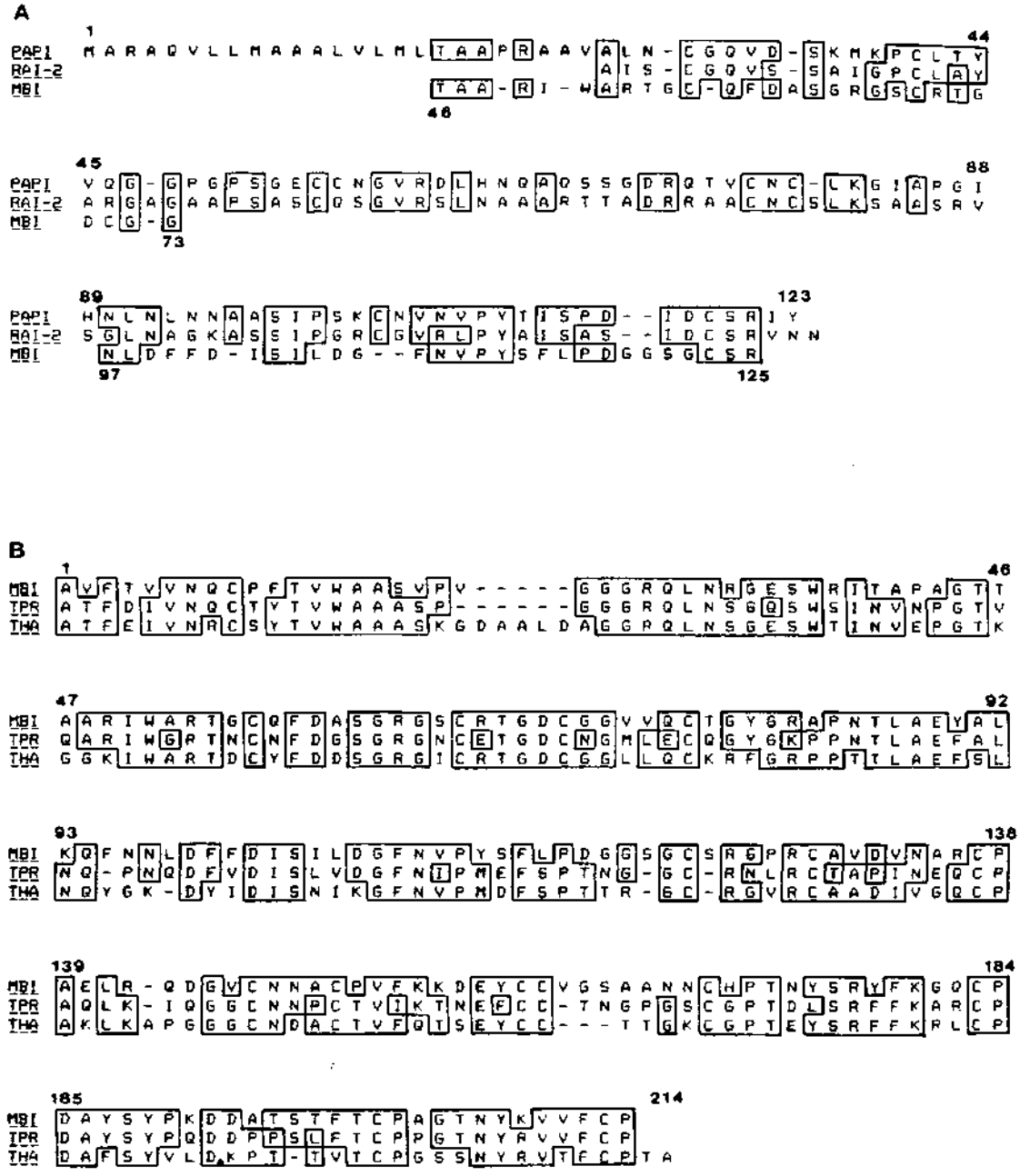

Figure. 9. a) Comparison of the complete sequences of PAPI, a probable α-amylase/proteinase inhibitor from barley (Mundy and Rogers, 1986), and RAI-2, the α-amylase inhibitor I-2 from ragi (Campos and Richardson, 1984), with homologous segments of MBI, a bifunctional α-amylase/trypsin inhibitor from maize (Richardson et al., 1987). b) Alignment of the complete sequence of MBI with those of thaumatin (THA) and a pathogenesis-related protein induced by tobacco mosaic virus (TPR), as reported by Richardson et al., 1987). Conserved positions are boxed.

Sequences of the highly homologous tomato and potato isoinhibitors have been aligned in Fig. 10. The structure of CPI-II from potato has been deduced by X-ray diffraction analysis at 2.5Å resolution of a complex of the inhibitor with carboxypeptidase A (Rees and Lipscomb, 1982). CPI-II, which has no helical structures or β-pleated sheets, binds to the enzyme like an extended substrate. The Val38-Gly39 peptide bond is cleaved and Gly39 gets trapped in the binding pocket of the enzyme. In the case of the tomato inhibitor, which lacks an amino acid at position 39, inhibition also takes place, indicating that this amino acid is not required for activity.

A significant increase of CPI was observed in the upper, unwounded leaves 38h following wounding of the lower leaves from potato plants and this response increased as the plants grew older (Hollander-Czytko et al., 1985). The levels of CPI accumulation in upper, unwounded leaves were increased by multiple wounds in a similar manner to that described for proteinase inhibitors I and II of potatoes. Finally, the inhibitor was also found to accumulate in the vacuoles of unwounded, wound-induced leaves (Hollander-Czytko et al., 1985).

INHIBITORS OF THIOL-PROTEINASES

Protein inhibitors of thiol-proteinases, such as papain, ficin, bromelain, etc., have been identified in different plant species, but only a limited number of studies have been devoted to them and it is not yet possible to classify them into structural families.

A family of isoinhibitors from pineapple stems that competitively inhibit papain, ficin, and endogenous bromelain, is probably among the best characterized of this group (Reddy et al., 1975). They consist of two chains with 41 and 10-11 amino acid residues respectively (Fig. 11). It has been proposed that the two chains, which are linked by disulphide bridges, are generated from single-chain precursors by excision of a "bridge" peptide which links the amino-terminal of the A chain to the carboxy-terminal of the B chain (Reddy et al., 1975).

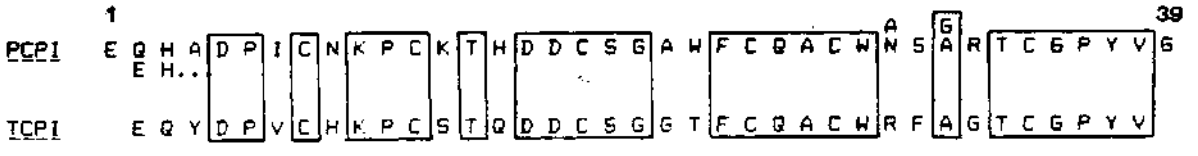

Figure. 10. Comparison of carboxypeptidase inhibitors from potatoes (PCPI) and tomatoes (TCPI) (see Hass and Ryan, 1981). PCPI has a variant that differs at the N-terminal as indicated in the figure. Conserved positions are boxed.

An inhibitor of M_r 24,000 from the seeds of the Bauhinia tree is active against a wide range of proteinases: papain, ficin, bromelain, trypsin, chymotrypsin and pepsin (Goldstein et al., 1973).

Two inhibitors from maize endosperm, with M_rs of about 13,000 and 9,500 respectively, have been found to be active against papain, ficin and bromelain (Abe et al., 1980), whereas an inhibitor isolated from dehulled rice seeds, which has an M_r of about 12,000, was only active against the first two enzymes (Abe and Arai, 1985). An unstable papain inhibitor has been purified from the grain of foxtail millet (Tashiro and Maki, 1986).

INHIBITORS OF CARBOXYL PROTEINASES

A polyvalent, heat stable inhibitor of M_r 27,000 which is active against cathepsin D, a carboxyl proteinase, has been isolated from potatoes (Keilova and Tomasek, 1976a,b). This inhibitor simultaneously inhibits trypsin and chymotrypsin and has no effect on hog and chicken pepsin, rennin, or cathepsin E. The inhibition of cathepsin D and trypsin is reversible and competitive, with Ki values of 3.8 x 10M and 8.6 x 10M, respectively.

Broad specificity proteinase inhibitors have been also characterized in cultured cells of *Scopolia japonica* (Solanaceae; Sakato et al., 1975). The inhibitors were shown to be polypeptides of M_r 4,000-6,000. They are potent inhibitors of pepsin, trypsin, chymotrypsin, plasmin and kallikrein, and had no effect on papain. These inhibitors form stable complexes with trypsin and chymotrypsin in an equimolar ratio. The inhibitory mechanism for both enzymes is non-competitive.

Figure.11. Sequences of the A and B chains of an inhibitor of thiol-proteinase from pineapple stem (Reddy et al., 1975).

INHIBITORS AND PLANT METABOLISM

Most of the described inhibitors are only active against heterologous enzymes and it is hard to envisage their involvement in metabolic processes of their respective species, with the possible exception of a storage role which has been postulated for them in view of their special accumulation in reserve tissues and the dependence of the concentration of some of them on nitrogen fertilization. Although such a role cannot be excluded, and might be even probable in some cases (especially in tissues like the cereal endosperm that are completely degraded during germination), in other cases a storage function is not likely because they are lost from the seed by diffusion (Tan-Wilson and Wilson, 1982) or undergo a specific proteolytic processing that seems to be different from the non-specific one of the true reserve proteins (Hartl et al., 1986; Yoshikawa et al., 1979b; Madden et al., 1985; Lorensen et al., 1981; Wilson and Chen, 1983).

Only those few inhibitors that have been shown to affect endogenous proteases and amylases are likely to play specific roles in plant metabolism.

Proteinase inhibitors that inhibit endogenous proteinases are found in barley (Mikola and Enari, 1970; Kirsi and Mikola, 1971), rice (Horiguchi and Kitagishi, 1971), wheat (Preston and Kruger, 1976), maize (Reed and Penner, 1976; Abe et al., 1980), mung beans (Baumgartner and Chrispeels, 1976), Scots pine (Salmia and Mikola, 1980; Salmia, 1980), and alfalfa (Gonnelli et al., 1985). At least some of these inhibitors might be specific for thiol-proteinases (see Ryan, 1981). They are usually present in small amounts and rapidly disappear during early stages of germination. The disappearance of inhibitory activity is accompanied by a rise of the endopeptidase activity, but the kinetics of the two processes, both in mung bean (Baumgartner and Chrispeels, 1976) and in Scots pine (Salmia, 1980), suggest that they are not causally related. Furthermore, since the endogenous proteinase and the inhibitor have different subcellular locations, these authors have postulated that the inhibitors do not control the breakdown of storage proteins by the enzyme associated with the protein bodies, but probably play a protective function with respect to other cellular components. As previously indicated, a possible role in the prevention of starch degradation has been proposed for the endogenous α-amylase inhibitors (Mundy et al., 1983), but as in the case of the endogenous proteinase inhibitors, no clear metabolic role has been fully demonstrated for them.

INHIBITORS AND PLANT PROTECTION

Several lines of evidence indicate that possibly a majority of the plant inhibitors that are active against heterologous proteinases and α-amylases play a protective role against the attacks of animal predators, insects, fungi, bacteria, and viruses. The inhibition of hydrolytic enzymes from these organisms may be directly toxic or even lethal to them, or could affect their fitness in such a way that different feeding habits might evolve. The action of some inhibitors on insect and microbial proteases has been repeatedly reported (Birk et al., 1963b; Applebaum et al., 1964a; Applebaum and Konijn, 1966; Peng and Black, 1976; Yoshikawa et al., 1976; Mosolov et al., 1976, 1979). Similarly, α-amylases of different origins have been tested against plant inhibitors (Applebaum et al., 1964b; Silano et al., 1975; Powers and Culbertson, 1982; Orlando et al., 1983; Baker, 1987). Particularly significant in the present context was the observation that insects which were able to feed on a tissue like the cereal endosperm, which has a high concentration of α-amylase inhibitors, had significantly higher α-amylase activities than those that were not able to do so (Silano et al., 1975). More recently, Gatehouse et al. (1986) have studied the effects on larvae development of inhibitor preparations from wheat endosperm that were strong inhibitors of digestive α-amylases from larvae of *Tribolium confusum*, a storage pest of wheat products, and *Collosobruchus maculatus*, a storage pest of legume seeds. While very high inhibitor concentrations were required to affect survival of larvae from the first species, quite low concentrations were effective against those of the second. The authors concluded that a detoxification mechanism must have been functioning in the first case, besides the considerably higher α-amylase activity, to account for the low sensitivity.

A more direct link of proteinase inhibitors with plant protection is represented by the already described systemic response which has been investigated by Ryan and co-workers. Increased levels of inhibitors are induced not only by the mechanical lesions caused by insects (see Ryan, 1984) but also following infection by fungi: Peng and Black (1976) demonstrated that while an incompatible race of *Phytophthora infestans* was able to elicit higher inhibitor levels in tomato plants, a compatible one produced either a small transitory increase or no increase. The systemic response was two times greater with the incompatible race than with compatible ones.

The effects of proteinase inhibitors on insects have been investigated in a number of cases. A possible involvement of these inhibitors in the protection of barley against grasshoppers was postulated because leaf extracts of the less sensitive variety tested had a considerably higher anti-chymotrypsin activity (Weiel and Hapner, 1976). Growth retardation and delayed pupation of corn borer larvae have been observed in feeding experiments with the Kunitz soybean trypsin inhibitor, while the maize trypsin inhibitor had no effect (Steffens et al., 1978). These and similar studies suggest but do not demonstrate a protective role for the inhibitors. More

conclusive evidence has been obtained for the resistance of the cowpea (*Vigna unguiculata*) to the bruchid beetle *Callosobruchus maculatus*. The only resistant variety found in a screening of over five thousand varieties had a higher level of proteinase inhibitors, whose antimetabolic nature was demonstrated in feeding trials (Gatehouse et al., 1979). In comparison, soybean trypsin inhibitor and lima been trypsin inhibitor were relatively ineffective (Gatehouse and Boulter, 1983). A convincing demonstration of the protective potential of the cowpea inhibitor has been obtained by cloning the corresponding gene and expressing it in tobacco under a constitutive promoter. According to a recent news item (Newmark, 1987), the transformed tobacco plants have been tested against bud- and army worms, and in both cases, they fail to grow and eventually die. This approach will allow in the near future testing the possible protective properties of different inhibitors.

An exciting new development concerns virus resistance. It seems that resistance to the cowpea mosaic virus in cultivar Arlington depends on the inhibition by a plant inhibitor of the thiol-proteinase encoded by the virus, thus preventing proteolytic processing of the viral polyprotein (see Ponz and Bruening, 1986). The recently reported homology of a PR-protein, induced by infection with tobacco mosaic virus, with a bifunctional inhibitor from maize (Richardson et al., 1987) is also of considerable interest in the present context.

INHIBITORS AND NUTRITION

Due to their abundance in many plant tissues, which are used as food and feed components, and to their relative stability versus heat, enzymatic degradation, or extreme pH, the potential incidence of proteinase and α-amylase inhibitors on human health and on animal production has been extensively investigated, leading to some controversies which are not quite settled yet.

The effect of trypsin inhibitors has been recently reviewed by Liener (1986), who has pointed out the need for further research to remove the remaining uncertainty concerning possible risks to humans. The pancreas of rats and chicks fed on raw soybeans is stimulated to produce more trypsin and becames enlarged as a result of hypertrophy and hyperplasia. The secretory activity is subject to inhibition by intraluminal trypsin through the inhibition of the release of the hormone cholecystokinin (CCK) from the intestinal mucosa. The inhibition of trypsin by the inhibitor results in an increased release of CCK (Green and Lyman, 1972; Liddle et al., 1984). Most of the information relating to the nutritional effects of trypsin inhibitors has come from experiments with animals and while the above described effects appear also in mouse, hamster and young guinea pig, besides the rat and the chick, other animals such as the dog, pig and calf do not display this sensitivity (see Liener, 1986). For this

reason, extrapolation to man is not easy, although it has been shown that raw soybeans can inhibit completely trypsin and chymotrypsin in human pancreatic juice (Krogdahl and Holm, 1981). The Kunitz inhibitor, which is heat labile, is inactivated by incubation with gastric juice, while the Bowman- Birk inhibitor is both heat stable and insensitive to gastric juice, and therefore, more likely to be present in processed soybean-containing foods and to survive passage through the stomach. The infusion of Bowman-Birk inhibitor into the duodenum of human subjects has been shown to significantly increase the enzyme content of the pancreatic juice (Goodale et al., 1985). A reported outbreak of gastrointestinal illness in individuals who had consumed underprocessed soy protein extender in a tuna fish salad would be a good illustration of the potential risk for humans (Gunn et al., 1980). In contrast, a potentially beneficial side-effect has been postulated for these inhibitors with the claim that diets containing soybeans or soybean protease inhibitors prevent the appearance of tumors in mouse skin treated with carcinogens, of breast tumors in rats subjected to ionizing radiations, and of spontaneous liver cancer in C3H mice (see Troll et al., 1984).

Inhibitors of salivary and pancreatic α-amylases in the diet could potentially affect starch digestion. Indeed, Puls and Keup (1973) proposed the use of the wheat α-amylase inhibitor as an antihyperglycaemic agent, based on tests carried out in rats, dogs, and humans. A large number of commercial preparations of α-amylase inhibitors or "starch blockers" obtained from kidney beans reached the market. Eventually, these products were discredited and their distribution discontinued on the basis of their ineffectiveness in humans (Carlson et al., 1983; Bo-Linn et al., 1982). A biochemical analysis of commercial starch blockers subsequently showed not only that these preparations were not particularly enriched in α-amylase inhibitors with respect to the crude meal, but that they contained trypsin inhibitors and lectins, as well as enough endogenous, inhibitor-insensitive α-amylase to offset their alleged effectiveness as inhibitors of starch digestion (Liener et al., 1984). More recent studies, using better characterized inhibitors, seem to confirm significant effects in rats and humans. An α-amylase inhibitor, prepared from black kidney beans (*Phaseolus vulgaris*), given to young rats by stomach tubing mixed with a starch meal was able to reduce in a dose-dependent manner the hyperglycaemia resulting from starch utilization (Lajolo et al., 1984). Similarly, a purified white bean inhibitor was found to be stable in human gastrointestinal secretions, to slow dietary starch digestion in vitro, and to rapidly inactivate amylase in the human intestinal lumen (Layer et al., 1985). The potential usefulness of α-amylase inhibitors in the treatment of diabetes and obesity cannot be inferred from these studies but deserves reconsideration.

ACKNOWLEDGEMENTS

Assistance from D. Lamoneda, and Grants No. 2022/83 and No. 0193/85 from the Comision Asesora de Investigacion Cientifica y Tecnica are gratefully acknowledged.

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